ON THE DIALYSABILITY OF THE GROWTH-ACTIVATING PRINCIPLE CONTAINED IN EXTRACTS OF EMBRYONIC TISSUES.

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Since it was first observed by Carrel and Burrows (1) in 1911 that extracts made from embryonic tissues had a great stimulating action upon the growth of cells under artificial cultivation, very little study has been devoted to the investigation of the nature of the substance responsible for this activity. Burrows has found evidence to show that this substance is formed by cells in proportion to the oxygen absorbed by them, and has termed it archusia (S) (2). In his recent book, Fischer (3), in addition to summarizing what is known regarding its physicochemical properties, describes a series of experiments with it which he undertook but from which he obtained very indefinite results. In fact it still remained obscure whether the so called activating substance was of a crystalloidal or colloidal nature. Consequently, with this in view it was decided to examine an active embryonic tissue extract by a process of dialysis in order to determine whether the active molecules concerned are capable of diffusing through a collodion membrane impermeable to proteins. Certain preliminary experiments on these lines which suggested that this might be so were carried out previously (4).

Method.

The method for the estimation of growth is essentially that described by Wright (4), though it was varied in some details.

The technique used was the ordinary hanging drop culture in a hollow ground slide. The cells used in the experiments were emigrant cells from the hearts of

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embryo chicks of between 8 and 11 days of incubation. Ebeling has found that these cells are second only to a permanently cultivated strain of fibroblasts in the uniformity of their rate of multiplication.

The medium used was obtained by mixing fowl plasma with an equal volume of a buffered saline solution[1] which was slightly modified from the one devised by Pannett and Compton (5).

For each series of experiments some twenty to thirty cultures of heart muscle were set up in this medium and were so cultivated for about 48 hours. At the end of this time they were examined microscopically, and those showing good halos of cells emigrating from the implanted fragment were selected and set aside for recultivation, the remainder being rejected.

In this way a number of cultures were obtained which possessed roughly the same number of emigrant cells and therefore were potentially equal from the point of view of subsequent growth. These selected slides were then recultured by the addition to each culture of a small droplet of the medium under examination. This droplet, which was roughly equal in volume to that of the plasma originally used to make the culture, was carefully spread by the aid of an iridectomy knife in a thin uniform layer over its surface. These slides were then reincubated for a further 18 to 20 hours, at the end of which time they were rapidly washed in saline and fixed in Bouin's solution. After staining in hematoxylin and mounting in balsam the preparations were examined under an oil immersion lens and the number of mitoses in each culture counted. In this way the extent of growth in each type of medium was determined.

The growth-stimulating media were obtained in the following way. Under sterile precautions chick embryos were removed from their shells, placed in Petri dishes, and there finely minced with scissors. The resulting pulp was transferred to a centrifuge tube and rotated rapidly for several minutes. The supernatant fluid was then removed with a pipette and diluted in the desired proportion with the saline solution previously described.

Dialysis was carried out through collodion membranes which were made with attention to the points emphasized by Gates (6). The collodion solution was

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[1] The preparation of the saline used was as follows:

Solution A.	NaCl	8.00	per cent.
	KCl	0.42	" "
	$CaCl_2$	0.20	" "
Solution B.	$Na_2HPO_412H_2O$	0.43	" "
	$NaH_2PO_4H_2O$	0.043	" "

8 cc. of Solution A were added to 88 cc. of freshly distilled water in an Ehrlenmeyer flask. 4 cc. of Solution B were measured into a second flask. After sterilization of both in the autoclave, Solution B was added to A with a sterile pipette. This constitutes the solution used. Its pH varied from 7.5 to 7.7.

obtained by dissolving 8 gm. of collodion in a mixture of 50 cc. each of ether and absolute alcohol. The actual membrane was prepared in the interior of a 100 cc. beaker, and after being allowed to drain for about 1 minute the beaker was filled with 95 per cent alcohol. After about 10 minutes this was replaced by water. The membrane, which then came free, was removed from the beaker and allowed to stand in distilled water.

For the process of dialysation one of these membranes was mounted on a glass ring about 4 cm. in diameter and 2½ cm. deep, being held in place by a rubber band. Actual dialysis took place in a small glass crystallizing dish about 4½ cm. in diameter, the ring and membrane being supported above the floor of the dish by a bent glass rod. The apparatus, set up in this form in a suitable protective container and containing a small quantity of distilled water, was then sterilized by autoclaving.

The extract used in the experiments was placed in the outer crystallizing dish so that should small tissue fragments be contained in it they should not fall upon the membrane and so mechanically impair diffusion. Dialysis was in every case carried out against the saline solution already described.

In these experiments three media had their growth-stimulating activity assessed: the *saline;* the *extract,* from the under side of the membrane, after dialysis had taken place; and the saline solution from the upper side of the membrane which consequently contains whatever substances have passed through it. This latter solution I shall refer to subsequently as the *diffusate.*

The biuret reaction was used to test for the presence or absence of proteins in the diffusate.

EXPERIMENTAL RESULTS.

Experiment 1.—The embryonic tissue extract was made from five 8 day old embryos and after preparation was diluted with saline to 40 per cent. 3 cc. of diluted extract were dialysed against 3 cc. of the saline for 72 hours. The biuret reaction was *negative.* The cells used were from a 9 day embryo.

No. of Mitotic Figures in Cultures.

Saline.	Extract.	Diffusate.
12	31	28
12	34	15
11	16	42
1	41	18
4	21	67
0	67	24
Average.....7±2	35±5	32±5

Experiment 2.—The embryonic tissue extract was made from five 10 day old embryos and after preparation was diluted with saline to 40 per cent. 6 cc. of diluted extract were dialysed against 6 cc. of the saline for 72 hours. The biuret reaction was *negative*. The cells used were from a 9 day embryo.

No. of Mitotic Figures in Cultures.

Saline.	Extract.	Diffusate.
Average.....2	24	26

Experiment 3.—The embryonic tissue extract was made from three 10 day old embryos and after preparation was diluted with saline to 50 per cent. 4 cc. of the diluted extract were dialysed against 4 cc. of the saline for 72 hours. The biuret reaction was *negative*. The cells used were from an 11 day embryo.

No. of Mitotic Figures in Cultures.

Saline.	Diffusate.
Average.....2±1	62±9

In this experiment a comparison was only made between saline and diffusate.

CONCLUSIONS.

These experiments appear to indicate that whatever substance in embryonic tissue extracts excites mitosis, and therefore would appear to be the growth-stimulating substance, is capable of passing through a collodion membrane which is impermeable to proteins, at all events in such concentration as would give rise to any biuret reaction. The substance is therefore diffusible.

I should like to express my deep appreciation of the great kindness and assistance I have received during my stay in Dr. M. T. Burrows' laboratory.

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THE RELATION BETWEEN ATHEROSCLEROSIS AND INGESTED CHOLESTEROL IN THE RABBIT.

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INTRODUCTION.

Earlier investigators who fed diets containing animal protein to herbivora described the occurrence of disease of the aorta that simulated human atherosclerosis. They attributed the experimental lesion to the cholesterol of the diet. Our own earlier experience[1] with diets of this general type led us to conclude that the large amount of protein and not the cholesterol had caused the lesion. We reached this opinion because the cholesterol content of our diet was smaller than the smallest amount alleged to have produced atherosclerosis in the hands of other workers. The data contained in the papers of the earlier workers seemed to us insufficient to prove that cholesterol was the primary factor, for one or more of the following reasons; *viz.*, that in the experiments of Steinbiss,[2] Saltykow,[3] Stuckey,[4] Anitschkow and Chalatow,[5] the duration of the feeding of cholesterol was not extensive enough; the cholesterol was administered to animals on diets which were abnormal and well might have led to metabolic disturbance under any condition; or the cholesterol was fed in conjunction with large amounts of protein. We felt, therefore, that further information was necessary to settle this point.

In order to get an entirely unequivocal answer to this question,

[1] Newburgh, L. H., and Clarkson, S., *Arch. Int. Med.*, 1923, xxxi, 653.

[2] Steinbiss, W., *Virchows Arch. path. Anat.*, 1913, ccxii, 152.

[3] Saltykow, S., *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 415.

[4] Stuckey, N. W., *Centr. allg. Path. u. path. Anat.*, 1911, xxii, 379; 1912, xxiii, 910.

[5] Anitschkow, N., and Chalatow, S., *Centr. allg. Path. u. path. Anat.*, 1913, xxiv, 1.

we determined to feed cholesterol in different amounts over long periods of time to rabbits that were receiving diets otherwise normal. In this way we hoped to determine: first, whether ingestion of cholesterol at any level is capable in itself of producing vascular disease; and if so, second, how large an amount is required to produce the lesion.

In earlier experiments[1] from this laboratory dealing with this question a diet containing 36 per cent protein was used. The diet resulted in a nitrogen metabolism of about 1 gm. per kilo, roughly four times the normal; and caused the rabbits to ingest from 17 to 31 mg. of cholesterol per kilo daily, according to the size of the animal. Such animals developed advanced intimal disease in 18 to 36 weeks. The atherosclerosis found in these animals was due either to excess of protein or of cholesterol in the diet. If cholesterol were the causative factor it should produce the lesion when added to a normal diet in the amount in which it is present in the above mentioned animal diet. Failure to obtain lesions under these conditions would demonstrate that cholesterol in such amounts was not productive of atherosclerosis; and would justify the conclusion that the excess of protein, which was the only other known abnormal factor, was responsible for the lesion.

The second question which we hoped to answer by means of the experiments to be described was whether cholesterol in any amounts would cause atherosclerosis when it was added to an otherwise normal diet.

When our work was partly completed it became evident that small amounts of cholesterol (such as occur in the high protein diet previously mentioned), when added to a normal diet, have no effect on the aorta or on the level of blood cholesterol. Accordingly, it seemed desirable to discover whether the high protein diet with its very marked effect upon the aorta would change also the blood cholesterol level. This seemed the more necessary after we found that the rabbit could be made to ingest cholesterol in amounts sufficient to induce both arterial disease and hypercholesterolemia.

Methods.

Care of the Animals.—The animals used for the experiments were from the laboratory stock. They had had the usual diet of bread, oats, hay, and greens.

They were from 2½ to 6 months old when treatment was begun. During the experiment they were kept in groups of from four to ten in pens with outside runways. Whenever an animal appeared to be infected it was removed from the series and discarded.

The rabbits were divided into five groups. The first group gave us the control series of blood cholesterols. These rabbits received the stock diet and were bled for the purpose of the experiment, or samples were obtained from the blood of rabbits that were being bled to death in order to supply blood for bacteriological purposes. The other four groups daily received 25, 113, 253, and 507 mg. respectively of cholesterol in capsules. It was the aim of the experiment to administer the amount of cholesterol corresponding to the quantity found in the high protein diet to the second group, and increasingly larger amounts to the other groups. However, it was necessary to regulate the dose to the size of the capsule obtainable. The smallest capsule used held 113 mg. of cholesterol, which was the nearest approximation to the desired eighth of a gram dose. Progressively larger capsules held 253 and 507 mg. respectively. For the first group there was no capsule small enough to hold exactly 25 mg. of cholesterol. Sugar of milk and the cholesterol were mixed in such proportions that a capsule of convenient size would hold the proper dose.[6]

In order to determine whether the ingestion of cholesterol had any effect on the blood level of cholesterol, samples of blood were drawn in some cases from the heart, in some from the ear.

5 cc. of the whole blood collected in an oxalated receptacle were pipetted into a flask containing an alcohol-ether mixture (3–1), with constant agitation. This was heated gently over a water bath for a few minutes, then set aside to cool to room temperature. When cool it was filtered. The filtrate then was made slightly alkaline to litmus with concentrated NaOH, and evaporated to dryness. The residue was taken up several times with hot chloroform. This in turn was evaporated to dryness and taken up with 10 cc. of 95 per cent alcohol, and heated. To this hot solution a 1 per cent digitonin solution was added. Usually only 40 mg. of digitonin were used. If on the addition of the digitonin a heavy precipitate came down quickly, more digitonin solution was added. This precipitate then was filtered on filter paper whose desiccated weight had been determined. The filter paper holding the digitonin cholesteride was then put into the desiccator until the weight became constant.[7]

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[6] To be sure that the rabbits actually got the cholesterol they were placed on their backs, the jaws were kept open by means of a piece of wood with a hole in the center which came just over the gullet, and the capsule was passed down the pharynx by means of a small rubber tube.

[7] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

DATA.

State of Aorta.—Group II.—Twenty rabbits were fed 25 mg. of cholesterol daily over a period lasting from 51 to 288 days. In no single instance did careful examination of the aortæ reveal an atherosclerotic process. (Table III.)

Group III.—This group received 113 mg. of cholesterol daily from 12 to 302 days. One of these nineteen rabbits, that had ingested cholesterol for 302 days, possessed an aorta which was the site of very slight atherosclerosis. (Table IV.)

Group IV.—Twenty-five rabbits received 253 mg. of cholesterol daily for a period of from 33 to 246 days. Thirteen were fed 147 to 246 days and eight (62 per cent) of the latter animals showed definite atherosclerosis. One other fed 71 days showed very slight abnormal areas which on microscopical examination were found to consist of slight thickening of the intima. Of twelve animals fed 33 to 125 days, eleven (92 per cent) had normal aortæ. When one compares the level of blood cholesterol with the state of the aorta (Table V), one finds that four of the eight atherosclerotic rabbits had abnormally high readings and that in the case of the other four the readings were all well within the normal.

Group V.—507 mg. This group included seven animals, five of which had well marked lesions, but two others showed lesions so minute that even histologically they could not be pronounced positive for atherosclerosis. This group was fed from 47 to 86 days. (Table VI.)

Lesions.—The lesions in the aortæ of rabbits fed cholesterol were identical with those produced by the ingestion of a high protein diet. Grossly the injury appeared as small, yellowish white, slightly raised streaks and plaques. In most instances it consisted of from one to four isolated little plaques, 1 to 3 mm. in diameter. This involvement occurred in the arch of the thoracic aorta, or at the exits of the larger vessels just below the arch. In five cases there were many scattering plaques from the arch nearly to the caudal bifurcation. In several instances the extent of the lesion grossly did not correspond to the microscopical findings; *i.e.*, in the case of one or two plaques of minute size we found the histological picture of an advanced process.

The involvement was primarily in the intima. To get some idea of the extent of involvement microscopically, we have sorted the atherosclerotic aortæ into three groups labeled slight, moderate, and extensive (Table I). Slight indicates that the lesion consists of only a few layers of fatty cells; moderate, the intimal thickening is maximal due to many layers of cells; extensive denotes not only cases of maximal thickening but also those with fibrosis on the lumen side of the lesion, and involvement of the media.

TABLE I.

Extent of Vascular Lesion.

Animal.	Daily dose.	Total dose.	Slight.	Moderate.	Extensive.	Blood cholesterol.
	mg.	*gm.*				*mg. per 100 cc.*
CH73	507.3	43.63		+		382.6
CH157	507.3	40.076		+		249.9
CH16	507.3	28.53		+		Lost.
CH14	507.3	24.25		+		"
CH22	507.3	24	+			"
CH128	253.65	62			+	168.23
CH123	253.65	62	+			65.15
CH116	253.65	56		+		67.3
CH140	253.65	47		+		72.9
CH120	253.65	45		+		Lost.
CH32	253.65	37			+	133.4
CH26	253.65	37	+			126.6
CH30	253.65	37	+			64.8
CH31	253.65	18	+			166.4
CH92	113.2	34		+		70.99

The two cases marked extensive showed a pronounced intimal involvement, in one animal with fibrosis on the lumen side (No. CH32), and in the other with some involvement of the media (No. CH128). Both of these cases occurred in the series receiving 253 mg. daily. There were eight animals which fell into the class designated moderate. Four of these belonged in the group receiving 507 mg. daily. The eighth animal was of the series which ate 113 mg. daily. In the class marked slight, there were five animals. One belonged to the group receiving 507 mg. daily. The other four belonged to the 253 mg. series.

Blood Cholesterol.—Table II contains readings from thirty-six controls. It will be seen that the free cholesterol in this series ranges from 35 to 125 mg. per 100 cc. of whole blood.

TABLE II.

Free Blood Cholesterol in Control Rabbits.

mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
125.44	82.04	67.09	56.15
116.68	77.79	67.09 (fasting).	51.4
116.68	77.79	64.42	51.04
105.9	77.79	64.18	50.7
93.35	75.9	62.2	44.6
87.03	71.1	60.28	43.49
85.57 (fasting).	70.19	59.02 (fasting).	38.8
83.38	69.58	57.6	35.39 (fasting).
83.14	68.55	56.64	

TABLE III.

Animals That Received 25 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH80	288	8	30.63	—
CH101	286	7.9	15.9	—
CH78	282	7.8	Lost.	—
CH112	266	7.5	"	—
CH110	266	7.5	76.8	—
CH84	262	7.2	Lost.	—
CH103	249	6.7	95.2	—
CH53	162	4	92.3	—
CH60	149	3.6	20.9	—
CH57	149	3.6	62.7	—
CH95	141	3.5	42.5	—
CH54	140	3.4	37.4	—
CH64	132	3.2	58.3	—
CH66	132	3.2	59.8	—
CH109	120	3	Lost.	—
CH87	104	2.5	23.9	—
CH56	76	1.9	Lost.	—
CH107	68	1.5	79.7	—
CH58	63	1.5	66.6	—
CH65	51	1.2	88	—

In Table III, the blood cholesterol of twenty animals receiving 25 mg. of cholesterol daily is tabulated in relation to the duration of the feeding. The range is from 16 to 95 mg.

Table IV contains the same information for the rabbits receiving 113.2 mg. daily, with a range of from 23 to 130 mg. The four slightly elevated readings in this group do not occur in the animals that received the largest doses of cholesterol. Furthermore, it should

TABLE IV.

Animals That Received 113.2 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH83	302	34	87.52	—
CH92	302	34	70.98	+
CH96	299	33	78.76	—
CH94	272	30	48.1	—
CH98	225	25	72.9	—
CH81	225	25	Lost.	—
CH108	225	25	22.7	—
CH104	225	25	51.5	—
CH100	225	25	129.8	—
CH35	164	18	Lost.	—
CH42	144	16	130.4	—
CH37	129	14.6	Lost.	—
CH39	127	14.3	128.5	—
CH36	84	9.5	126.1	—
CH34	78	8.8	Lost.	—
CH38	69	7.8	56.5	—
CH41	67	7.4	42.88	—
CH40	60	6.8	77.98	—
CH43	12	1.35	85.08	—

be noted that the animal showing atherosclerosis had a blood cholesterol well within the normal limits.

Table V: The range for this group is from 52 to 251 mg. Of the eighteen readings in Table V there are seven higher than the highest control; but of these seven abnormally high figures, six occurred in animals that were fed less than 150 days, whereas only one high reading is found among ten rabbits fed from 160 to 246 days.

Eight atherosclerotic rabbits in this series had blood cholesterols

determined at the end of the experiment. Four of these showed high readings; *i.e.*, 168, 133, 126, 166, whereas the other four had readings well within the normal; *i.e.*, 65, 67, 72, 64 mg. of free cholesterol per 100 cc. of whole blood. Among the four animals which had high cholesterol levels, two showed lesions of the aorta which were

TABLE V.

Animals That Received 253.65 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH128	246	62	168.22	+
CH123	246	62	65.15	+
CH129	231	58	70.01	—
CH116	222	56	67.3	+
CH115	209	53	52.2	—
CH127	203	51	Lost.	—
CH140	187	47	72.93	+
CH132	187	47	69.04	—
CH120	181	45	Lost.	+
CH131	159	40	103.1	—
CH32	147	37	133.4	+
CH26	147	37	126.6	+
CH30	147	37	64.88	+
CH33	125	31.7	136.18	—
CH136	111	28	98.2	—
CH135	99	25	251.1	—
CH137	80	20.29	Lost.	—
CH31	71	18	166.4	+
CH24	69	17.5	53.19	—
CH122	66	16	Lost.	—
CH124	54	13	63.69	—
CH118	54	13	127.3	—
CH125	46	11.66	Lost.	—
CH25	44	11.1	"	—
CH130	33	8.3	"	—

slight, and two showed extensive lesions. Two other rabbits in this series whose blood cholesterol was abnormally high showed no intimal lesions. It is noteworthy that Rabbit CH135 presented a normal aorta even though the blood cholesterol is recorded at 251 mg. (the highest in the series), whereas Rabbit CH123 with a blood cholesterol of only 65 mg. was found to have an atherosclerotic aorta.

Table VI shows that the ingestion of 500 mg. of cholesterol daily produces a marked hypercholesterolemia. The range here is from 250 to 383 mg.

High Protein Rabbits.—Since the above data make it clear that the cholesterol contained in the high protein diet is not the cause of the

TABLE VI.

Animals That Received 507.3 Mg. Cholesterol Daily.

Animal.	No. of days of feeding.	Total dose.	Blood cholesterol.	Atherosclerosis.
		gm.	*mg. per 100 cc.*	
CH75	87	44.14	286.5	—
CH73	86	43.63	382.6	+
CH157	79	40.08	249.91	+
CH16	55	28.53	Lost.	+
CH19	50	25	"	—
CH22	48	24	"	+
CH14	47	24.25	"	+

TABLE VII.

High Protein Diet.

Relation of the Diet to the Blood Cholesterol and the Vascular Lesion.

Animal.	Blood cholesterol before feeding.	Blood cholesterol at end of experiment.	Atherosclerosis.
	mg. per 100 cc.	*mg. per 100 cc.*	
CH146	51.04	238.24	Moderate.
CH141	77.79	215.32	Extensive.
CH142	64.18	189.62	Moderate.
CH145	64.42	180.00	"
CH144	67.09	126.89	Absent.
CH147	56.64	87.52	"
CH149	83.38	68.07	"
CH150	77.79	36.95	"

vascular lesion produced by this diet it seemed desirable to determine whether this diet had any effect on the blood cholesterol. Accordingly, we fed eight rabbits the high protein diet containing 36 per cent of protein prepared in the same way as that previously used to produce atherosclerosis. A determination of the blood cholesterol was made

in the case of each animal just before it was placed on the experimental diet. After the animals had been on the diet for 6 months second blood cholesterol readings were obtained and the rabbits then were killed so that the aortæ might be examined. Table VII shows the blood cholesterol readings at the beginning and again at the end of the experiment and the condition of the aorta in the case of each animal. It will be seen that the initial blood cholesterols were all well within the normal range. After a period of 6 months half of the rabbits showed blood cholesterol readings very much higher than the normal range. These same rabbits were without exception atherosclerotic, but the other half of the rabbits had normal aortæ and in them the blood cholesterols all fell within the normal range.

DISCUSSION.

In our earlier paper we discussed in detail the work of previous investigators who had studied the effect of various diets on the production of atherosclerosis, and pointed out reasons for not accepting the conclusions of those who believed that they had demonstrated that an excess of cholesterol in the diet was the etiological factor concerned.[8]

We accordingly have planned our experiments with the intention of

[8] Bailey (Bailey, C. H., *J. Exp. Med.*, 1916, xxiii, 69) fed six rabbits cholesterol dissolved in cottonseed oil added to a daily ration of crushed barley. "On an occasional day greens were given with the barley." The daily dose of cholesterol varied from 10 to 500 mg., but the total period of feeding was brief, in no instance lasting as long as 100 days. He found that the rabbit which received 100 mg. daily for 83 days had moderate intimal lesions; one that received 500 mg. daily for 36 days had moderate intimal lesions; one that received from 100 to 500 mg. daily for 97 days had pronounced intimal lesions; and one rabbit that had received cottonseed oil without cholesterol for a longer time than any of the cholesterol-fed rabbits had no vascular disease. It is unfortunate that Bailey did not add the cholesterol to a diet more nearly approaching that which may be considered normal for a rabbit; for one is unable to avoid the question whether sustaining the rabbits on crushed barley in itself may not have given rise to an abnormal metabolic condition. Furthermore, his experiments are too few and too brief to answer the question with which we are mainly concerned; *i.e.*, whether the presence of relatively small amounts of cholesterol such as occur in meat-containing diets when ingested over long periods of time are responsible in any way for the vascular lesions regularly found in rabbits that are sustained by such diets.

getting data adequate to determine the relation of ingested cholesterol to atherosclerosis. We have found that the addition of some 10 to 15 mg. of cholesterol per kilo daily to otherwise strictly normal diets is not productive of vascular disease even though fed over a period considerably longer than that required to produce atherosclerosis with great regularity by means of a diet rich in beef muscle with its accompanying cholesterol. The following calculations will make it clear that the cholesterol of this meat diet is present in amounts which cause rabbits fed this diet to ingest, daily, cholesterol to the extent of 10 to 15 mg. per kilo; *i.e.*, amounts of cholesterol comparable with those which we fed to rabbits of Group II with uniformly negative results.

The urinary nitrogens of two rabbits on this 36 per cent protein diet were determined. The urine was collected for 7 days. Rabbit CH141 weighing 3100 gm. put out 2160 cc. urine, which yielded 25.4 gm. of nitrogen or 3.6 gm. per day. This was at the rate of 1.1 gm. of nitrogen per kilo. Multiplying 3.6 by 6.25 gives 22.5 or the number of gm. of protein ingested by the rabbit daily. Our analysis of different samples of the desiccated beef used showed that it contained 7 mg. of cholesterol per 5 gm.[9] Therefore, in the 22.5 gm. of protein eaten daily, this rabbit at the same time absorbed 31.5 mg. of cholesterol. Rabbit CH149 whose urinary nitrogen was determined also, weighed 2.2 kilos and put out 1.99 gm. of nitrogen per day or 0.9 gm. per kilo. This animal ate 12.5 gm. of protein daily and therefore consumed 17.5 mg. of cholesterol with the meat diet.[10]

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[9] Windaus quoting Neumeister (Windaus, A., *Arch. Pharm.*, 1908, ccxlvi, 117), states that dried muscle contains 0.23 per cent cholesterol.

The analysis of seven different samples of beef gave a mean of 7 mg. of cholesterol for 5 gm. of beef.

Sample	I	5 gm.	yielded	11.7	mg.
"	II	5 "	"	10.6	"
"	III	5 "	"	5.6	"
"	IV	5 "	"	7.5	"
"	V	5 "	"	4.7	"
"	VI	5 "	"	6.1	"
"	VII	5 "	"	3	"
		Mean =		7	mg.

[10] The rabbits were kept in a metabolism cage for 7 days, and the urine collected during the entire period. The bottom of the cage and fine wire matting on which the rabbit rested were covered with a paraffin-ether mixture (10 gm. paraffin were mixed with 8 ounces of ether). The urine was collected in a receptacle that was white and 5 cc. of 3½ per cent HCl were kept in it. Methyl red was

After the fact had been established that these small amounts of cholesterol are without effect upon the rabbit aorta, the next experiments were planned to determine the minimum daily dose of cholesterol when fed for 6 months or more, which would produce atherosclerosis.

We chose the period of 6 months since we previously had learned that our high protein diets caused atherosclerosis within this period of time. Accordingly another series of rabbits was fed 113 mg. daily. Nine of these rabbits took this dose of cholesterol for 9 or more months, but only one of them was found to have a small atherosclerotic patch. Even if this lesion is attributed to the cholesterol in the diet, it is evident that the ingestion of 113 mg. of cholesterol by rabbits, for long periods of time, is harmful to the aorta only as a rare exception. When, however, 250 mg. were fed daily vascular lesions were obtained in nine out of twenty-five rabbits. Thirteen rabbits were fed this dose from 147 to 246 days. Of these, eight (62 per cent) showed vascular disease. Finally, when the daily dose was 507 mg. atherosclerosis was obtained in five of the seven rabbits fed for the comparatively short period lasting from 47 to 87 days. It should be noted that a majority (71 per cent) of the rabbits which received the 500 mg. dose developed aortic disease within a period of time during which the animals receiving 250 mg. of cholesterol rarely developed vascular injury. Specifically, in a series of twelve rabbits

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used as the indicator. A few crystals of thymol were kept in the urine container also. As the urine was voided it was put into a stoppered bottle with some HCl in it, and kept on the ice. The bottom of the cage and the wire matting were rinsed with distilled water into the container. A piece of gauze (two thicknesses) was placed in the bottom of a funnel and the urine and rinse water poured through this. All large particles were retained by the gauze. A few bits of the high protein food were found in the urine container. Since there was a possibility that they might add to the nitrogen content of the urine, a few small pieces of the food were shaken with distilled water, filtered, and then tested for albumin. The test failed to give the albumin reaction. Furthermore, some particles were put into a flask, a few drops of the HCl used for the urine and a few drops of distilled water added. This was allowed to stand overnight at room temperature. The test for albumin again was negative. It seems evident from this that whatever of the food may have reached the urine container was not digested far enough to give a test for albumin and therefore could not add any nitrogen to that of the urine.

that received 250 mg. from 33 to 125 days, there was only one whose aorta was abnormal.

A comparison of the state of the aorta with the daily ingestion of cholesterol, and with the total amount of cholesterol ingested, gives a much closer relationship between the daily dose and atherosclerosis than between the total dose and atherosclerosis. This becomes evident if one selects animals from the third, fourth, and fifth groups, each of which has ingested approximately a total of 25 gm. of cholesterol, and compares the state of their aortæ with the daily dose of

TABLE VIII.

Relation between Size of the Daily Dose and the Intimal Injury.

Animal.	Daily dose.	Total dose.	Atherosclerosis.
	mg.	*gm.*	
CH19	507.3	25	—
CH14	507.3	24.25	+
CH22	507.3	24	+
CH33	253.65	31.7	—
CH136	253.65	28	—
CH135	253.65	25	—
CH137	253.65	20.29	—
CH31	253.65	18	+
CH94	113.2	30	—
CH98	113.2	25	—
CH81	113.2	25	—
CH108	113.2	25	—
CH104	113.2	25	—
CH100	113.2	25	—
CH35	113.2	18	—

cholesterol (Table VIII). By so doing it will be found that 75 per cent of the rabbits of this type (25 gm.) that belong in the 500 mg. series have aortic disease; that 20 per cent of the rabbits in the 253 mg. series which received about 25 gm. as a total dose, were positive; while none of the seven in the 113 mg. series which totalled 25 gm. or thereabouts was positive. A large daily dose quickly produces atherosclerosis. A small daily dose within the time limit of the experiment never produces atherosclerosis regardless of the total amount ingested.

These feeding experiments studied in connection with the earlier high protein series indicate that alimentary atherosclerosis may be produced in rabbits by adding to the diet either an excess of protein or an excess of cholesterol, but the excess of protein necessary to obtain this result is very much less than that necessary for cholesterol. Rabbits whose nitrogen metabolism was 1.1 gm. per kilo, which roughly is four times the normal,[11] became atherosclerotic in 6 months to a year, whereas the rabbit that normally eats no cholesterol[12] must ingest 250 mg. daily to become atherosclerotic.

Lesions.

We already have pointed out that vascular disease caused by ingestion of cholesterol differs in no way from that found in animals fed a high protein diet, and corresponds to the descriptions found in textbooks dealing with disease of the human aortic intima. We use the term atherosclerosis to designate a primary lesion of the intima. "The most conspicuous change is a thickening of the intima accompanied by fat deposits."[13] The process never was far enough advanced in any of our animals to present calcification. Before attributing the aortic lesions under discussion to the cholesterol in the diet, it is necessary to know whether lesions of this type occur spontaneously in the rabbit. The examination of the aortæ of 100 rabbits from the laboratory stock which were killed for the purpose of examination, or were used for bleeding and then examined, never has revealed an example of primary intimal involvement.

However, a certain type of abnormality was not infrequent. This lesion always occurred in the region of the arch. It was uniformly small, being about 1½ mm. in diameter. It was a very white, slightly

[11] The urinary nitrogen of a normal rabbit was determined. It weighed 2.15 kilos and put out 2710 cc. of urine. This amount yielded 3.68 gm. for 7 days, or 0.24 gm. per kilo.

[12] According to the following calculations, a rabbit normally eats about 80 mg. of phytosterol daily. 3 gm. of hay yielded 0.5 mg. of phytosterol. A rabbit eats about 113 gm. of hay. 5 gm. of oats yielded 1.3 mg. of phytosterol. A rabbit eats approximately 225 gm. There are no published data indicating that the ingestion of phytosterol has an effect on the aorta.

[13] MacCallum, W. G., *Physiol. Rev.*, 1922, ii, 70.

raised process with a depression in its center. On microscopic study of this area it was seen to consist of a degenerative change in the media, unaccompanied by fat but often showing marked calcification. This spontaneous lesion corresponds closely to a similar condition found in human arteries as described by Mönckeberg. While this type of lesion sometimes was found in rabbits which in addition showed disease of the intima, the distinction between the spontaneous lesion of the media and the experimental lesion of the intima always was clear and we were satisfied that every rabbit labelled atherosclerotic had developed the experimental lesion regardless of whether the spontaneous medial lesion was present or absent. Hence, we conclude that spontaneous aortic disease is not a source of error in this study.

Blood.

One would expect *a priori* that the ingestion of cholesterol in amounts sufficient to damage the aorta would produce the deleterious effect through the path of hypercholesterolemia. In order to get information on this point a large number of blood cholesterol determinations were made by the Windaus method, on control animals and on animals that had received the various levels of cholesterol for many weeks. Inspection of Table II will show that normal rabbits exhibit a wide range of blood cholesterol readings—from 35.4 to 125.4 mg. with a mean of 71.3 mg. for 100 cc. of whole blood. The animals that received 25 mg. daily had bloods all well within the normal limits, with a mean of 56.7 mg. In the group of animals that received 113 mg. of cholesterol daily, the mean was 80.65 mg. In this series there were four readings just above the highest control, but none of these occurred in those animals that received cholesterol for the longest periods of time; and we accordingly are unable to show that this order of dosage is productive of hypercholesterolemia. Furthermore, it should be pointed out that the only animal of this group with an atherosclerotic aorta had a blood cholesterol of 71 mg. With the animals receiving 253 mg. it again was impossible to establish a relationship between the duration of the feeding and the number of mg. of cholesterol in the blood. Only one of the six animals that were fed more than 200 days had an abnormally high reading. On the other

hand, the highest reading in the whole group occurred in a rabbit that was fed but 99 days. In addition to this, atherosclerotic aortæ were found in animals that had normal as well as high readings, and the animal that exhibited the most extreme hypercholesterolemia had a normal aorta. It would appear then to be true that the prolonged ingestion by rabbits of 250 mg. of pure cholesterol daily does not result in general in hypercholesterolemia; and conversely, that such animals may have a marked hypercholesterolemia without disease of the aorta. When animals receive 500 mg. daily, a well marked hypercholesterolemia occurs. Since five of the seven animals became

TABLE IX.

Effect of Single Dose of Cholesterol on the Blood Level of Cholesterol.

Animal.	Cholesterol ingested.	Blood cholesterol.				
		Before ingestion.	After ingestion.			
			3 hrs.	6 hrs.	12 hrs.	24 hrs.
	gm.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
A	0.5	87.03	56.71			
C	0.5	116.7		119.6	104.3	
G	0.5	67.1 Fasting.		67.3	49.6	
E	0.5	35.4 Fasting.		54.5	44.5	
B	2 empty capsules.	106	117.2	97.3	89	93.4

atherosclerotic (moderately so in four cases) it may be true that there exists a causal relationship between the cholesterol in the blood and the state of the vessel; but we have not sufficient data dealing with this question to be in a position to give a final answer.[14]

Since there is so little correspondence between the amounts of cholesterol ingested and the level of blood cholesterol, we tried to determine what effect if any was produced by the ingestion of a single

[14] In an unpublished communication of Miss Kunde and Professor Carlson of the University of Chicago we learn that the blood of cretin rabbits may run 100 per cent above normal in cholesterol content without any indication of arteriosclerosis.

large dose of cholesterol on the level of the blood for the following 24 hours. Reference to Table IX shows that the blood values were uninfluenced by a single ingestion.[15]

High Protein Rabbits.

The final group of rabbits to which the high protein diet was fed brought out the fact that this diet with a cholesterol content in itself far too small to affect the blood cholesterol does produce marked hypercholesterolemia in those rabbits that become atherosclerotic as a result of the diet. We thus have evidence that this particular type of hypercholesterolemia has its origin in some mechanism other than that of absorption from the digestive tract, and the question immediately arises whether the atherosclerosis is caused by the hypercholesterolemia in this series of rabbits, or whether the two abnormalities occur concommitantly and do not bear the relation of cause and effect to each other, or finally whether the aortic disease occasions the hypercholesterolemia. Since both abnormalities always have occurred together in these experiments it would seem that they bear an intimate relation to each other, but our present information does not permit us to explain this relationship further.

CONCLUSIONS.

1. The range of free cholesterol in the blood of rabbits, as determined by the Windaus method, varies from 35 to 125 mg. with a mean of 71 mg. per 100 cc. of blood.

2. The small amount of cholesterol contained in the high protein diet used by us in earlier work and causing atherosclerosis does not affect the blood cholesterol nor does it cause arterial disease.

3. In order to produce atherosclerosis it is necessary to feed at least ten times that amount of cholesterol.

4. In rabbits receiving such amounts both hypercholesterolemia and atherosclerosis occur, but it is not possible to establish any close

[15] The only other observations in this field which we could find are those of Lehman (Lehman, E. P., *J. Biol. Chem.*, 1913–14, xvi, 495), who claims that a single dose of ingested cholesterol produces cholesterolemia in rabbits, but his published data do not appear to support his contention.

parallelism between the two. High blood readings are found in rabbits with normal aortæ and atherosclerotic rabbits in this series sometimes have shown a normal blood cholesterol.

5. With still greater doses of cholesterol one finally reaches an amount which regularly produces hypercholesterolemia and atherosclerosis within a few weeks.

6. A new series of rabbits fed the high protein diet shows that those rabbits which become atherosclerotic also develop hypercholesterolemia. We attribute this elevation of the blood cholesterol to a metabolic disturbance directly referable to the excess of protein in the diet and not to its cholesterol content.

The writers wish to acknowledge their appreciation of the suggestions offered by the late Dr. Carl C. Warden when the work was begun, and without which it could not have been carried through.

THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

I. The Rôle of the Reticulo-Endothelial System in the Production of Diphtheria Antitoxin.

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INTRODUCTION.

The significance of the reticulo-endothelial system for the production of antibodies has recently aroused a great deal of interest. The early investigations into the production of antibodies which have been directed to the study of the leucocyte-forming organs, such as spleen, bone marrow, lymph nodes (1), or the glands of internal secretion, (testicle, thyroid) (2), as the source of the immune bodies, almost uniformly failed to prove any unmistakable relationship, although they did establish the fundamental importance of these organs in this process. More recent experiments on the reaction of the reticulo-endothelial system in immunity processes seem to point the way toward a better understanding of these hitherto obscure phenomena. It appears that the wide distribution of this particular tissue throughout the body and its general occurrence in the hematopoietic and lymphatic system, including the spleen and liver, would help to correlate the facts already observed and would also furnish a better explanation of many of the phenomena of general and local immunity.

Experience has shown that the reticulo-endothelial system, distinguished by Aschoff (3, 4) as a separate unit because of its ability selectively to take up and retain foreign particulate matter, can be effectively eliminated physiologically, in certain experimental animals, without inducing disproportionate, non-specific by-symptoms, by the intravenous injection of some indifferent colloid (such as India ink, various dyestuffs, or metallic salts). Many workers have used this blocking experiment in conjunction with splenectomy or without this procedure

as a criterion for determining the extent to which the reticulo-endothelial system participates in the production of the various antibodies. Subsequent to the early work of Murata (5), in 1918, as quoted by Aschoff (4), the significance of the reticulo-endothelial system, especially of its elements in the spleen, for the action of antigens in the body, was made the subject of extensive research by Bieling and Isaac (6). Experiments on the production of hemolysins in mice, in which these authors used the blockade together with splenectomy, suggested to them that the reticulo-endothelial system as a whole and, in particular, that part which is found in the spleen ranked foremost as the site of antibody production following the immunization with corpuscular antigenic material. Rosenthal and Fischer (7), in repeating this work with rabbits that were blocked and splenectomized, observed no inhibition of hemolysin formation, while Siegmund (8) found that the production of hemolysins and hemagglutinins was lessened or totally suppressed in highly blocked and splenectomized rabbits. Standenath (9), in testing precipitin formation in rabbits after the use of only moderate doses of India ink, found that it was increased rather than diminished, while splenectomized rabbits showed a lower titer. Another indeterminate observation was made by Fränkel and Grunenberg (10), who studied the formation of agglutinins against *proteus* X-19 in rabbits blocked with "elektroferrol." They found the agglutinin titer of the serum in blocked animals to be not materially lower than the titer of controls. Vannucci (11), on the other hand, reported the decrease of agglutinin formation subsequent to the injection of carmine and Wasserblau dye. Gay and Clark (12) in a recent paper report the average difference in the titer of hemolytic amboceptors produced in blocked rats and rabbits and in normal controls as 1:75 and assert that similar results are seen in precipitin formation. Paschkis (13) also regards the reticulo-endothelial system, especially that found in the spleen, as the site of antibody production and believes that a functional elimination of this system by blocking is possible. Kobayashi and Shiwotsu (14) studied the formation of typhoid agglutinins in rabbits which had been (*a*) injected with iron somatose, (*b*) splenectomized, (*c*) blocked and splenectomized. In agreement with Bieling, they found that agglutinin production was only partially inhibited by either blocking or removal of the spleen alone but practically no agglutination titer was observed in immunized rabbits in which blocking was combined with splenectomy. By following the response of treated animals to repeated injections of antigen, they found that a marked repair of the disturbed function took place, probably by compensation through other allied tissues. Neufeld and Meyer (15) observed that blocked and splenectomized mice frequently could not be actively immunized against pneumococci, while passive immunization in such animals is not interfered with. In a very interesting paper Singer and Adler (16) were able to show that a rabbit highly immunized against Pneumococcus Type III behaved just as a non-immune control animal, after the injection of India ink.

These various statements in the literature seem to indicate that by blocking the reticulo-endothelial system of experimental animals the

appearance of various antibodies (precipitins, agglutinins, hemolysins) was frequently prevented or delayed, which observations were still more distinct if the spleen was removed simultaneously, shortly before immunization.

In view of the somewhat conflicting reports in the literature, it is obviously important that further investigations in this field should be carried on. It would seem that the nature and dose of blocking material, method of administration, quality of antigen, species of experimental animal, and time interval between completion of the blockade and antibody determination, influence largely the outcome of the experiment. No work has as yet been reported in which the production of antitoxins in the blocked animal has been studied.

Walbum and Madsen (17) succeeded in increasing the antitoxic titer of the serum of horses by the intravenous injection of various metallic salts, and their results have since been confirmed by a number of other investigators. Neufeld (15) interprets these results as illustrating the opposite phase of the problem in question; namely, that very small amounts of colloidal material may induce an irritation rather than a blockade of the reticulo-endothelial system, with a subsequent increase of antibodies in the circulation (see also Standenath (9)). Experiments on the site of antibody formation would seem better grounded if the fate of antigenic material in the body were known to some extent. Wadsworth and Vories (18) have been able to show that neither the leucocytes of the dog nor those of the guinea pig neutralize or combine with diphtheria or tetanus toxin. Furthermore, they found in these experiments that, although brain tissue combines with and neutralizes tetanus toxin, as established by the work of Wassermann and Takaki (19), it has no action on diphtheria toxin. On the other hand, it appears from the work of Wadsworth and Hoppe (20) that diphtheria toxin and culture filtrates from a number of organisms had a definitely inhibitory action on the phagocytosis of leucocytes. This action was not affected by specific antitoxin nor antibacterial serums. This work clearly distinguished a substance which inhibited phagocytosis quite apart from the toxin—the toxin having no demonstrable action on these cells and suffering no loss of potency in their presence. Other workers have directed their attention to the hematopoietic organs, certain sessile cells of which are known to display a strong phagocytic activity. Their observations suggest that the bacterial toxins, such as diphtheria toxin and tetanus toxin, are fixed and absorbed by the spleen and apparently by the reticulo-endothelial system (Aschoff (3, 4), Bieling (21), Bieling and Gottschalk (22)).

A study of antitoxin production in animals in which the reticulo-endothelial system has been partially or wholly blocked would thus offer a promising field for investigation. Moreover, such a study

would have certain technical advantages as compared with the titration of other antibodies: the neutralization of diphtheria toxin by antitoxin, as carried out by the intracutaneous test, is delicate enough to show with sufficient accuracy any slight differences in the titer of antibody. Also, it would make possible the repeated determination of the antitoxin content in the tissues of the original experimental animal without recourse to tests in other animals or to *in vitro* titrations.

The occurrence of active and passive anaphylaxis in the blocked animal will be presented in a later paper.

EXPERIMENTAL WORK.

The study recently undertaken in this laboratory had for its object the comparison of diphtheria antitoxin production in guinea pigs, the reticulo-endothelial system of which had been blocked with India ink, with antitoxin production in normal, control animals, following a subcutaneous injection of diphtheria toxin-antitoxin mixture. To avoid repetition the first group will hereafter be referred to as blocked animals. By preliminary work it had to be determined, first, how much India ink could safely be given at one time to a guinea pig of a certain weight, and, second, how many injections had to be given to accomplish as complete a saturation of the reticulo-endothelial system as was consistent with the well-being of the animal. In much of the earlier work devoted to study of various factors in antibody formation, the animals were subjected to somewhat radical procedures (extensive surgical operations, injection of highly toxic substances, radiation, etc.) which in themselves constituted a serious interference with the physiological functions of life. In our experiments, we strove to attain an elimination of the reticulo-endothelial system by means of saturation with India ink that would not materially otherwise depress the vitality of the animal. It was found that the maximum dose of ink[1] that could safely be injected intravenously into a guinea pig of between 250 and 300 gm. weight was from 1.5 to 2 cc. of a 1:5 dilution with physiological salt solution. After such doses the

[1] The ink used was a commercial brand of India ink (insoluble) manufactured by Charles M. Higgins and Company, Brooklyn, New York.

animals remained perfectly well and did not show any symptoms of emaciation. By means of histological examinations it was found, furthermore, that two such injections following each other at short intervals would fill a large percentage of the reticular elements in the spleen and liver. These organs looked somewhat enlarged in the first days after these injections and had assumed an entirely black color. The bone marrow, also, looked grayish. Deposits of ink were found, besides, in the retrosternal lymph glands. Neither kidneys, nor adrenals, nor lungs seemed to be materially affected. In animals killed

TABLE I.

Determination of a Suitable Dose of India Ink for Blocking Injections.

Guinea pig No.*	Injections† (1:5 ink dilution).		Total volume injected.	Amount of concentrated ink injected.	Results.
	May 7	May 10			
	cc.	*cc.*	*cc.*	*cc.*	
1	2 2		4	0.8	Died in 2 hrs.
2	2 1		3	0.6	Very ill.
3	2	2	4	0.8	Slightly ill after second injection.
4	2	1.5	3.5	0.7	Very slightly ill after second injection.
5	1.5	1.5	3	0.6	No symptoms.
6	1.5	1.5	3	0.6	" "

* Animals weighed from 240 to 270 gm.

† Injections were given intravenously into the leg vein.

2 weeks after the completion of the blockade, the liver and spleen were very much reduced in size and were of hard consistency, apparently due to cirrhotic processes in which large areas of the parenchyma were replaced by connective tissue.

The method used for intravenous injections was that fully described in 1918 by Shiga (23) and later recommended by Roth (24, 25), who evidently had not seen Shiga's work on the subject, since he makes no mention of it in either of his two papers. Table I illustrates the finding of the maximum tolerance of guinea pigs for India ink.

After the dose of ink suitable for blocking had been thus determined, six guinea pigs, constituting the first experimental series, were blocked with two intravenous

injections of a 1:5 dilution of ink (1.5 to 2 cc.) on 2 different days; 2 days after the last blocking injection, they received 1 cc. of diphtheria toxin-antitoxin mixture[2] by subcutaneous injection. Simultaneously three normal, control guinea pigs were immunized with the same dose. After an interval of 3 weeks all nine animals

TABLE II.

Antitoxin Production in Maximally Blocked and in Normal Guinea Pigs Immunized with Diphtheria Toxin-Antitoxin Mixture.

Guinea pig No.	Weight of animals.	Blocking injections* (1:5 ink dilution).		Immunizing injections† (toxin-antitoxin mixture 140).		Results of 1st intracutaneous test Aug. 1.			Results of 2nd intracutaneous test Aug. 8.			Result of subcutaneous test Aug. 15.
		Date.	Dose.	Date.	Dose.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	2 M.F.D. per 250 gm. body weight.
	gm.		*cc.*		*cc.*							
7	250	July 4	1.5	July 11	1	+	+	+	−	−	−	Survived.
		" 9	1.5									
8	250	" 4	1.5	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
9	260	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
10	300	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
11	300	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
12	240	" 4	1.5	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
13	260			" 11	1	−	−	+	−	−	−	"
14	250			" 11	1	−	−	+	−	−	−	"
15	275			" 11	1	−	−	Sl. +	−	−	−	"
16	260					+	+	+	+	+	+	Died.

* All blocking injections were given intravenously.

† All immunizing injections were given subcutaneously.

were tested by intracutaneous injections of 1/150, 1/100, and 1/50 M.F.D. of diphtheria toxin for the degree of immunity developed. Also, a normal guinea pig

[2] The mixture used was the 1/10 L+ dose of underneutralized diphtheria toxin-antitoxin mixture prepared and distributed as a routine product by this laboratory. This was used because it was a safer, quicker, and better known method of immunizing very susceptible animals.

that had been neither blocked nor immunized was tested as a control of the toxin dilutions. Readings were made each day for the first 3 days, then not again until the 5th and 7th days. A reaction was called positive only when a necrosis was observed on the 5th day. After another week, *i.e.* 4 weeks after immunization with the toxin-antitoxin mixture was begun, the intracutaneous injections were repeated on the other side of the animal. This method of testing the potency of toxin-antitoxin mixture follows closely the procedure described by Glenny (26)

TABLE III.

Antitoxin Production in Partially Blocked and in Normal Guinea Pigs Immunized with Diphtheria Toxin-Antitoxin Mixture.

Guinea pig No.	Weight of animals.	Blocking injections.*		Immunizing injections† (toxin-antitoxin mixture 140).		Results of 1st intracutaneous test Aug. 3.			Results of 2nd intracutaneous test Aug. 10.			Result of subcutaneous test Aug. 17.
		Date.	Dose.	Date.	Dose.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	2 M.F.D. per 250 gm. body weight.
	gm.		*cc.*		*cc.*							
17	250	July 13	1.5‡	July 13	1	+	+	+	−	−	−	Survived.
18	240	" 13	1.5‡	" 13	1	−	+	+	−	−	−	"
19	260	" 13	2‡	" 13	1	−	+	+	−	−	−	"
20	250	" 13	2‡	" 13	1	−	+	+	−	−	−	"
21	275	" 11	1§	" 13	1	−	+	+	−	−	−	"
22	240	" 11	1§	" 13	1	+	+	+	−	−	−	"
23	280	" 11	1§	" 13	1	−	−	+	−	−	−	"
24	250	" 11	1§	" 13	1	−	+	+	−	−	−	"
25	250			" 13	1	−	−	+	−	−	−	"
26	260			" 13	1	−	−	Sl. +	−	−	−	"

* All blocking injections were given intravenously.

† All immunizing injections were given subcutaneously.

‡ A 1:5 ink dilution was used.

§ A 1:20 ink dilution was used.

and his associates. The series was concluded by testing all the animals after another week had elapsed, with a subcutaneous injection of 2 M.F.D. of diphtheria toxin per 250 gm. body weight for the establishment of general immunity. The results of this series, which are given in Table II, will be discussed below in connection with those of the following experiment.

Another series, consisting of eight guinea pigs injected with ink and of two normal controls, was run, with the object of studying in four animals the effect

of only one blocking dose (1.5 or 2 cc. of a 1:5 ink dilution), followed with practically no interval (from about 3 to 5 minutes) by the immunizing injection, and in the remaining four the effect of smaller doses of ink (1 cc. of a 1:20 ink dilution). Table III illustrates this work.

DISCUSSION.

Since the usual methods of investigating antibody formation depend necessarily upon the determination of the presence of antibody in the circulation or tissue fluid (which evidently also applies to intracutaneous tests), it is impossible with the tests employed to distinguish between the production of antibody in the cells of certain tissues and its presence in the body fluids. *A priori*, any inhibitory effect on the antibody titer, of blocking may be attributed to a delay in the appearance of the antibody in the circulation due to changes of permeability in the cell membranes as well as to a cellular underproduction of it. Conclusions as to the actual site of antibody formation, drawn from such experiments, are only indirect and, unless extensively controlled, can only bring out a more or less intimate causal relation of the reticulo-endothelial tissue to the production of immune bodies.

It appears from Tables I to III that, by giving a sufficient amount of India ink intravenously to guinea pigs shortly before immunization with diphtheria toxin-antitoxin mixture, a delay of antitoxin production for about 1 week, as compared with normal immunized controls, can be demonstrated. Tested 3 weeks after the immunizing injections, the blocked animals reacted, without exception, positively to intracutaneous injections of 1/150, 1/100, and 1/50 M.F.D. of diphtheria toxin, behaving at this stage just like non-immunized animals, while immunized unblocked control animals reacted positively only to 1/50 M.F.D. This deficiency in antitoxin production, however, is only temporary and is compensated for by another very quick rise in antitoxin production occurring in the following week, so that at the end of 4 weeks both blocked and non-blocked animals reacted negatively to 1/50 M.F.D. The degree of immunity eventually attained in both groups is similar, as evidenced by the survival of all animals after the subcutaneous injection of 2 M.F.D. From comparison of the two series it must be concluded that for the depression of antitoxin production the dose of blocking material is of greater importance than the duration of the interval between blocking and immunization.

Finally, and contrary to Neufeld's supposition, it was shown that the small doses of ink employed in these experiments (about 1/12 of the blocking dose used in the first series) did not have a stimulating effect, but proved likewise to exert some inhibitory action on the production of antitoxin. This failure of smaller ink doses to exert a stimulating effect might be due to the fact that the doses used were not sufficiently small to bring about the physiological irritation.

The authors realize that the results obtained in this study must be interpreted with reserve in view of the fact that the antigen used was a complex substance, the action of which in inducing active immunity may not be as simple a process as that of toxin alone.

CONCLUSIONS.

1. Following massive doses of India ink injected intravenously into guinea pigs before a subcutaneous injection of diphtheria toxin-antitoxin mixture, no antitoxin was found in the blood serum for 3 weeks, as indicated by intracutaneous tests, whereas an appreciable amount could be detected in non-blocked, immunized control animals.

2. During the 4th week following immunization, the titer of the serum of blocked animals equaled that of non-blocked controls within the limits of the intracutaneous test dose.

3. The smaller doses of India ink used in these experiments, given before immunization, had no stimulating effect on the production of diphtheria antitoxin but, on the contrary, also inhibited the appearance of this antibody, although to a less extent.

We wish to express to Dr. Augustus B. Wadsworth our sincere gratitude for his most helpful suggestions and criticism. We are also indebted to Dr. L. J. Early for his cooperation in carrying out the histological work.

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STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

V. The Relation of Virulence to the Pneumococcidal Activity of Normal Rabbit Serum-Leucocyte Mixtures.

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The present paper deals with a further study of the pneumococcidal action of normal serum-leucocyte mixtures. Results of previous work (1), in which it was shown that the blood (serum and leucocytes) of certain pneumococcus-resistant animals possessed destructive properties for pneumococci, not found in the blood of susceptible animals, suggested that natural immunity to pneumococcus infection depends chiefly, if not entirely, on pneumococcidal activity of the blood. In these studies, pneumococci of high virulence[1] only were employed, and the animals tested represented well marked examples of natural immunity and susceptibility, the dog and cat on one hand and the rabbit and guinea pig on the other. It is a very commonly observed fact that within a single species of any of the usual laboratory animals there occur wide variations in susceptibility toward different strains of disease-producing pneumococci, and that among the so called susceptible animals such variations may be extreme. But why rabbits, for example, should succumb to 0.0000001 cc. of a culture of one strain of pneumococcus and resist successfully 0.1 cc. of another, has not been satisfactorily explained. The view that avirulence is to be accounted for by a failure of the microorganism to adapt itself to growth in the animal body does not seem to hold as a general explanation of this phenomenon, since pneumococci freshly isolated from cases of lobar pneumonia are found not infrequently to be of low pathogenicity for the rabbit and the guinea pig. Resistance against invasion by such

[1] The word virulence is used here to indicate a high degree of invasiveness for the so called susceptible animals, rabbit, mouse, and to a less extent the guinea pig.

an organism would seem to be more than passive in nature. The following experiments were undertaken with the purpose of determining whether the immunity shown by a relatively susceptible animal, as the rabbit, against certain strains of pneumococci, is associated with demonstrable pneumococcus-destroying powers in the blood.

Earlier work on this subject consisted chiefly in testing the bactericidal properties of blood serum or plasma, alone or in the presence of leucocytes, against pneumococci of low virulence for the animal species. The ingredients of the test were mixed in the test-tube or capillary pipette, incubated, then cultured for surviving organisms. Results varied widely but it is generally agreed that these experiments failed to show conclusively the presence of pneumococcus-destroying properties in the blood. More recently Heist and Solis-Cohen (2) studied this question by means of seeding pneumococci of varying virulence for the rabbit into whole uncoagulated rabbit blood, which was then drawn up into capillary pipettes and incubated. They found that the ability of a given strain of pneumococci to grow in rabbit blood was directly proportional to the virulence of the strain, and furthermore, that the failure of avirulent organisms to grow was due to the pneumococcidal action of the blood. However Bull and Bartual (3), in a series of carefully controlled experiments, were unable to confirm Heist and Solis-Cohen's findings. For a detailed discussion of the technique of these tests the reader is referred to the second paper of this series (1).

Technique.

Rabbits were employed throughout.[2] The growth inhibition tests were carried out as before (1). Pneumococci of Types I, II, II atypical, and III were tested. The rabbit leucocytes were obtained from an aleuronat pleural exudate on the day of experiment. Normal rabbit serum was procured from the animal supplying the leucocytes. These rabbits averaged 1500 to 1600 gm. in weight but occasionally larger ones were used.

For the virulence tests, young and actively growing cultures, with an opacity approximately equal to that of a "standard suspension" (4), were employed. Dilutions were made in meat infusion broth, pH 7.8. For each strain of pneumococcus a series of 3 to 4 rabbits was injected intraperitoneally with varying amounts of culture and observed for a period of 10 to 14 days. The rabbits were normal, full grown, and as nearly of the same weight as obtainable. When there were differences of weight in the series, the larger rabbits were given the larger doses of organisms. Repetition of these tests gave essentially the same results in all cases.

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[2] Growth inhibition tests with the serum and leucocytes of guinea pig were found to be unsatisfactory for this purpose, apparently because of the greater susceptibility to injury of guinea pig leucocytes during washing.

EXPERIMENTAL.

Experiment 1.—(Table I.) Two types of pneumococcus of different virulence were used. The Pneumococcus Type I was originally isolated from a case of lobar pneumonia and kept in blood broth for 6 years with an occasional animal passage. 0.000001 cc. of culture killed rabbits of 1600 gm. in 24 hours. The Pneumococcus Type II was obtained from the Hospital of The Rockefeller Institute some months previously and is referred to as D39/21. After five passages

TABLE I.

Comparison of a Virulent Pneumococcus Type I with an Avirulent Type II.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	Minimum lethel dose of culture.
	cc.				*cc.*
Avirulent Pneumococcus Type II.	0.01	+	+		
	0.001	0	+		
	0.0001	0	0	0	0.01
	0.00001	0	0	0	
	0.000001	0	0	0	
Virulent Pneumococcus Type I.	0.01	+	+		
	0.001	+	+		
	0.0001	+	+		
	0.00001	+	+		
	0.000001	+	+		0.000001
Controls with serum only.					
Avirulent Type II.	0.0000001		+		
	0.0000001		+		
Virulent Type I.	0.0000001		+		
	0.0000001		+		

through rabbits, its killing dose was found to be 0.01 cc. of the standard culture for rabbits of about 1500 gm. Agitation of the small tubes was maintained at twenty-four revolutions per minute continuously for 24 hours. The tubes were then left in the incubator without agitation for another 48 hours, at the end of which time smears were made from their contents with aseptic precautions. Those tubes showing no organisms in stained smear were cultured in dextrose blood broth.

It will be seen in Table I that the growth of the Pneumococcus Type II of low virulence (for rabbits) was inhibited to a marked degree,

while the highly virulent Type I grew out in the smallest numbers used. Tests were next carried out to determine whether similar differences between virulent and avirulent organisms were to be found in other types and strains of pneumococci.

Experiment 2.—(Table II.) Two types of pneumococcus were used: a Type II of low virulence (employed in Experiment 1), and a Type II atypical of high

TABLE II.

Comparison of Virulent Pneumococcus Type II Atypical with an Avirulent Type II.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	Minimum lethal dose of culture.
	cc.				*cc.*
Avirulent Pneumococcus Type II.	0.01	+	+		
	0.001	0	+		
	0.0001	0	0	0	0.01
	0.00001	0	0	0	
	0.000001	0	0	0	
	0.0000001	0	0	0	
Virulent Pneumococcus Type II atypical.	0.01	+	+		
	0.001	+	+		
	0.0001	+	+		
	0.00001	+	+		0.00001
	0.000001	+	+		
	0.0000001	+	+		
Controls with serum only.					
Avirulent Type II.	0.0000001		+		
	0.0000001		+		
Virulent Type II atypical.	0.0000001		+		
	0.0000001		+		

virulence for rabbits, obtained from the blood of a case of lobar pneumonia. Its minimum lethal dose was 0.00001 cc. of the standard culture for rabbits of 1350 gm. The test was carried out exactly as in Experiment 1.

The outcome of Experiment 2 (Table II) is similar to the finding in Experiment 1. The rabbit serum-leucocyte mixture failed to inhibit the growth of even a minute number of the virulent Type II atypical

pneumococcus but showed a marked bactericidal action for the strain possessing only a slight virulence for rabbits.

Growth inhibition tests were performed with a number of strains of Pneumococcus Type II, several of which had been isolated freshly from cases of lobar pneumonia. There was marked inhibition of growth in every instance and all these strains were found to be of low virulence for rabbits. On the contrary, all the Type I strains studied have been found to possess high virulence for rabbits and they grew readily in rabbit serum-leucocyte mixtures.

Tests performed with Type III pneumoccoci of varying virulence for rabbits gave, in general, the same results as were found with Types I and II, except that the bactericidal action of the serum-leucocyte mixtures for them was not as marked; *i.e.*, given a Type II and a Type III of about equally low virulence, the growth of Type II was inhibited to a considerably greater degree than was that of Type III. Consequently the difference between the virulent and the avirulent Type III organisms as tested in this way was less than that observed in the above experiments with Types I and II.

It was then considered desirable to determine whether enhancement of virulence by animal passage would result in a corresponding increase in the ability of the organism to grow in rabbit serum-leucocyte mixtures. Accordingly, attempts were made to increase the virulence of several stock strains of Types II and III, including those used in the foregoing experiments, but without success. A long series of animal passages in rapid succession was employed. In certain cases passage through mice and guinea pigs as well as rabbits was resorted to. Direct injection of heart's blood was also used. But at the end of 20 to 40 passages, the virulence for rabbits of the several strains was found to be only slightly increased. Comparative growth inhibition tests on these strains before and after animal passage showed no essential difference between them. This again strongly suggests that the pneumococcidal properties of the rabbit's blood reveals the state of the animal's resistance to the pneumococcus in question.

Another means of determining the relation between the pneumococcidal properties of the blood and susceptibility to pneumococcus infection was to be found in comparing the serum-leucocyte mixtures of full grown and young rabbits, since the latter are much more

susceptible to pneumococcus infection. It was ascertained that certain strains of Pneumococcus Type II, relatively avirulent for adult rabbits, killed young rabbits in minute doses. In the following experiment the growth ability of such a Type II was tested in the serum and leucocytes of a large and a small rabbit respectively.

TABLE III.

Comparison of Serum-Leucocyte Mixtures of the Full Grown and Young Rabbit.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	Minimum lethal dose of culture.
	cc.				*cc.*
Serum and leucocytes of full grown rabbit (1650 gm.).	0.01	+	+		
	0.001	0	0	0	
	0.0001	0	0	0	
	0.00001	0	0	0	0.01
	0.000001	0	0	0	
	0.0000001	0	0	0	
Serum and leucocytes of young rabbit (620 gm.).	0.01	+	+		
	0.001	+	+		
	0.0001	+	+		
	0.00001	0	+		0.00001
	0.000001	0	+		
	0.0000001	0	+		
Controls with serum only.					
Full grown rabbit.	0.0000001		+		
	0.0000001		+		
Young rabbit.	0.0000001		+		
	0.0000001		+		

Experiment 3.—(Table III.) The Pneumococcus Type II used was the same strain employed in Experiment 1. Its minimum lethal dose was 0.01 cc. of the standard culture for rabbits of 1500 gm. and 0.00001 cc. for rabbits of 650 gm. Leucocytes and serum were obtained from a large rabbit weighing 1650 gm. and a small rabbit weighing 620 gm. Two tests were set up at the same time. The technique of the test was as before.

The difference between the pneumococcidal activity of serum and leucocytes of large and small rabbits is strikingly shown in Table III.

While quantities of the standard suspension less than 0.01 cc. grew out in the adult rabbit serum-leucocyte mixtures, 0.0000001 cc. grew out in the serum and leucocytes of the young rabbit. Thus the development of pneumococcidal power in the blood appears to accompany the increased resistance to pneumococcus infection shown by the full grown rabbit. While complete studies of the parallelism between the acquisition of pneumococcus immunity and blood pneumococcidal activity by the growing rabbit have not been made, it has been found that the half grown rabbit of 1000 gm. in weight possesses both these properties to a considerable degree but not as marked as in the adult animal.

DISCUSSION.

The results of the above experiments suggest that the more susceptible animals, of which the rabbit is a type, possess the same kind of defense mechanism against pneumococcus infection as do the highly pneumococcus-resistant animals; *e.g.*, dog and cat. In examples of both classes of animal the blood has been shown to exert a marked destructive action on pneumococci of low virulence for the species studied. However, the differences in blood pneumococcidal power, between these animals so widely separated in degree of susceptibility to the highly virulent strains of pneumococci, are pronounced. This is shown by the fact that the serum-leucocyte mixtures of the dog or cat are capable of destroying virulent pneumococci in large numbers, whereas the serum and leucocytes of the rabbit are found to be without bactericidal activity for them. But whether the greater potency of the blood elements of the highly resistant animal is due to a higher concentration of the same pneumococcus-destroying properties occurring in the blood of the relatively susceptible animal, or is due to some added element, the foregoing experiments do not make clear. An analysis of this reaction will be dealt with in a subsequent communication.

The absence of pneumococcidal properties in the blood of the very young rabbit and the development of such properties during growth, would seem to account for the marked resistance shown by the adult rabbit against pneumococci of high fatality for the young animal. There may be other unknown factors, such as possible differences in

susceptibility of adult and young tissue cells to the products of pneumococcus growth, which act in conjunction with the pneumococcus-destroying powers of the blood as codeterminants of susceptibility and resistance. Yet even if such accessory factors do play a part, the ability of the body to destroy pneumococci is probably still of chief importance in preventing infection.

The direct parallelism between virulence for rabbits and ability to grow in rabbit serum-leucocyte mixtures, points to the possession by the virulent pneumococcus of a specific property capable of neutralizing the pneumococcidal action of the serum and leucocytes. There is reason to assume also that it is the lack of this same property, or its possession to a much less degree, which renders the avirulent pneumococcus susceptible to destruction by serum-leucocyte action, since the culture medium provided by the fresh serum alone, or by leucocytes and inactivated serum, is an excellent one for the growth of such organisms. These findings help to narrow the study of pneumococcus virulence to an investigation of the different reactions which may occur between the pneumococcus and its products, and the serum and leucocytes. The results of further observations of this nature are presented in the following paper.

SUMMARY.

Employing a method, described in an earlier publication, for testing the pneumococcidal activity of serum-leucocyte mixtures, a study has been made of the pneumococcus-destroying properties of the blood of a relatively susceptible animal, the rabbit, for pneumococci of low virulence for the species. It was found that rabbit serum-leucocyte mixtures possessed the power to kill avirulent pneumococci in relatively large numbers but failed to inhibit the growth of virulent organisms even in minute quantities. The results of numerous experiments in which all three types of pneumococci were employed indicated that the ability of a strain of pneumococcus to grow in rabbit blood is dependent on its virulence for the rabbit. The extreme susceptibility of the very young rabbit to strains of pneumococcus of low virulence for the full grown animal, was found to be associated with an absence of pneumococcidal properties in the blood of the young rabbit. These findings suggest that the relatively susceptible animals

possess the same type of defense mechanism against pneumococcus infection as do the highly pneumococcus-resistant species.

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STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

VI. The Specific Effect of Pneumococcus Soluble Substance on the Growth of Pneumococci in Normal Serum-Leucocyte Mixtures.

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Of recent years, investigation of the problem of virulence has been concerned chiefly with the effects of certain secretory products of the bacterial cell in relation to invasiveness. In the earlier literature, much attention was devoted to capsule formation, and observations from numerous workers showed that the development of a capsule is undoubtedly associated with increased virulence and resistance to phagocytosis in many bacterial species. The capsule was thought of generally as a protective envelope shielding the bacterium from injurious influences by virtue of its physical or physical-chemical properties. In the light of present knowledge, it seems probable that the presence of a capsule is indicative of great protoplasmic activity resulting in the elaboration of substances capable of neutralizing the defensive forces of the body. Even in those pathogenic microorganisms which do not show capsule formation, there have been observed evidences of ectoplasmic change, such as enlargement or swelling, during periods when the bacterium was actively engaged in its struggle against the body's defenses. It may be, as Zinsser (1) suggests, that the capsulated bacterium holds a large part of its neutralizing substance in a protective layer about itself, while the virulent non-capsulated species gives off its secreted substances, in chief part or entirely, to the surrounding medium.

In 1905 Bail (2) demonstrated in the peritoneal exudate of animals infected with virulent bacteria, a substance which when injected together with the same organism rendered a non-fatal dose, fatal. He called this substance "aggressin." Later Rosenow (3) showed that by suspending virulent pneumococci in normal salt

solution, and incubating the suspension for 48 hours, the supernatant fluid after centrifugation, contained a substance which neutralized the opsonins in normal human serum, rendered avirulent pneumococci resistant to phagocytosis, and produced an increase in their virulence for animals. To this substance he gave the name "virulin." He further showed that this antiopsonic action of the pneumococcal "virulin" is highly specific for pneumococci. It did not prevent the phagocytosis of streptococci and staphylococci.

The conclusions of the above authors that these substances, "aggressin" and "virulin," represented secretory products of the bacterial cell have been criticized on the ground that they contained extracts of the bacteria and hence their action might be due largely, if not entirely, to their endotoxin content.

With the discovery of the soluble specific substance of pneumococcus by Dochez and Avery (4) in 1917, the elaboration of which, as was shown, does not depend upon the solution or disintegration of the bacterial cells, but represents purely an extracellular product, attempts were made to determine whether or not the substance increases the virulence of pneumococci when injected into animals. So far, the results have been negative.

Briefly, the nature of the pneumococcus soluble substance is as follows: Dochez and Avery (4) showed that when pneumococci are grown in fluid media, there is elaborated into the cultural fluid a substance which precipitates with specific antipneumococcus serum. This substance is demonstrable in the cell-free filtrates of young pneumococcus cultures, and is also found in the blood and urine of experimentally infected animals and in the body fluids of patients suffering from lobar pneumonia. It is type-specific, not destroyed by boiling, by precipitation with alcohol, acetone, or ether, or by trypsin digestion. Recently, with a method of concentration and purification of the substance from pneumococcus cultures, Heidelberger and Avery (5, 6) have been able to obtain the soluble specific substance in a highly purified form. Analyses of material thus obtained showed that the substance consists mainly of carbohydrate, a polysaccharide derivative. It is non-antigenic (7) but type-specific and highly reactive. A dilution as high as 1:5,000,000 gives precipitation when mixed with homologous antipneumococcus serum.

In the preceding paper of this series (8), it was shown that the ability of pneumococci to grow in normal rabbit serum-leucocyte mixtures depended upon their virulence for rabbits. The growth of avirulent strains was inhibited, whereas virulent strains grew readily in this medium. In view of the fact that the individual elements employed in this test were present in known quantity and limited in amount, it was felt that specific effects of the soluble substance, which had not been shown to occur *in vivo* might be demonstrable under these conditions which are capable of control in a manner not possible in the animal body.

Materials.

Specific Soluble Substance.—The specific soluble substances of Type II (Preparation 20) and Type III (Preparation 31) pneumococcus were kindly provided by Drs. Avery and Heidelberger. The purified powder of each type was dissolved 1:50 in gelatin-Locke's solution which had been previously adjusted with $NaHCO_3$ to pH 7.4. The solutions were then sterilized by boiling in a water bath for 15 minutes, and kept in the refrigerator as stocks from which dilutions were made as needed.

Strains of Pneumococci Used.—Two strains of pneumococci were used. One was a Type II pneumococcus, which was relatively avirulent for rabbits and cats. 5 cc. of an actively growing broth culture failed to kill either the rabbit or cat. The other was a Type III pneumococcus to which the cat was also resistant. 2 cc. of the culture of this organism failed to kill.

Broth Filtrates.—In Experiments 4 and 5, broth culture filtrates were employed in place of the isolated specific soluble substances. The Type II broth filtrate was prepared from a culture of Pneumococcus Type II grown in meat infusion broth. While the organisms were still actively growing, the culture was centrifuged at high speed and the supernatant broth filtered through a Berkefeld filter. Precipitin test done on this filtrate gave a positive reaction to a dilution of 1:20. The Type III broth filtrate was similarly prepared. This filtrate gave a positive precipitin reaction in a dilution as high as 1:40.

Leucocytes and Serum.—Leucocytes used in all the experiments were obtained entirely from aleuronat pleural exudate, and the serum from the same animal furnishing the leucocytes. These were procured, in every case, on the day of the test.

Technique.

The method described previously (9) for demonstrating the growth-inhibitory and bactericidal action of normal serum-leucocyte mixtures for the pneumococcus was employed in all the following experiments. The technique given in that communication was closely adhered to.

In making up dilutions of pneumococcus soluble substances or broth filtrates, gelatin-Locke's solution which had been previously adjusted with $NaHCO_3$ to pH 7.4 was employed. On the other hand, gelatin-Locke's adjusted to pH 7.8 was used for diluting the bacterial suspensions. All dilutions were made immediately before the test was put up.

To the tubes that contained no soluble substance or broth filtrate, an equivalent volume of gelatin-Locke's solution was added.

The rate of agitation and oscillation was the same throughout all experiments, agitation 23 to 24, and oscillation 19 to 20, per minute. The agitation was kept up continuously for 72 hours; at the end of that time color changes were recorded and stained films of the contents of each tube were carefully examined for pneumococci.

TABLE I.

Rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + soluble specific substance 0.1 cc. + pneumococcus suspension 0.1 cc.

Pneumococci Type II. Amount of standard suspension.	Soluble specific substance. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
	Type II.		
cc.			
0.0001		++++	+
0.00001		0	0
0.000001		0	0
0.0000001		0	0
0.000001	1:2,500	++++	+
0.0000001	"	++++	+
0.0001	1:25,000, 1:250,000,	++++	+
0.00001	1:2,500,000	++++	+
0.000001	respectively.	++++	+
0.0000001		++++	+
0.0001	1:25,000,000	++++	+
0.00001	"	++++	+
0.000001	"	++++	+
0.0000001	"	0	0
0.0001	1:250,000,000	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
	Type III.		
0.0001	1:2,500	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
Controls without leucocytes.			
0.0000001	0		+
0.0000001	Type II S*1:2,500		+
0.0000001	Type III S 1:2,500		+
Plating	No. of colonies.		
0.0000001	103		
0.0000001	111		

* S = Soluble specific substance.

In every instance, the experiments were repeated several times on different days, with practically identical results.

EXPERIMENTAL.

Experiment 1.—(Table I.) Rabbit serum and rabbit leucocytes employed. Pneumococcus Type II and the soluble specific substances of Pneumococcus Types II and III were used as shown in the protocol. Control plates on the bacterial suspension were made with 1 per cent dextrose, rabbit blood agar.

It was found that this strain of Pneumococcus Type II of low virulence for rabbits, seeded into normal rabbit serum-leucocyte mixtures, failed to grow in quantities less than 0.0001 cc. of the standard suspension (Table I). However, the addition to the same serum-leucocyte mixture of a very minute amount of the Type II soluble specific substance, 1:2,500,000 dilution, caused growth of the pneumococci in all tubes. Growth occurred even in those that were seeded with as small a number of pneumococci as 0.0000001 cc. of the standard suspension. On further dilution to 1:25,000,000, the soluble substance still showed a marked growth-favoring effect, though not quite so great as the lower dilutions. At a dilution of 1:250,000,000, this action of the soluble substance disappeared and the normal growth inhibition again occurred. On the contrary, no such effect was obtained when a Type III soluble substance was used in place of the Type II substance, even employing a concentration as high as 1:2500. The degree of growth inhibition, it will be seen, was exactly the same as that in which no soluble specific substance was present. Controls on the bacterial suspension showed that the pneumococci were in excellent condition at the time when the experiment was set up, and in about the right calculated numbers.

In order to ascertain whether the soluble specific substance exerts the same action on the growth of pneumococci in serum-leucocyte mixtures of animals possessing a much higher degree of natural resistance to pneumococcus infection than the rabbit, the following experiment was performed.

Experiment 2.—(Table II.) Cat serum and cat leucocytes used. Other procedures were identical with those of Experiment 1.

As shown in Table II, the addition of a small amount of soluble substance of the homologous type to the cat serum-leucocyte mixtures

abolished completely their growth-inhibitory power. The fact that the soluble substance was not effective in as high dilution in the cat as in the rabbit serum-leucocyte mixtures is probably to be accounted for by the greater initial growth-inhibitory action of the former (compare Tables I and II). The specific action of the soluble substance was

TABLE II.

Cat serum 0.2 cc. + cat leucocyte suspension 0.1 cc. + soluble specific substance 0.1 cc. + pneumococcus suspension 0.1 cc.

Pneumococci Type II. Amount of standard suspension.	Soluble specific substance. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
	Type II.		
cc.			
0.01		++++	+
0.001		0	0
0.0001		0	0
0.00001		0	0
0.000001		0	0
0.000001	1:250	++++	+
0.0000001	"	++++	+
0.000001	1:2,500	++++	+
0.0000001	"	++++	+
0.001	1:25,000	++++	+
0.0001	"	++++	+
0.00001	"	++++	+
0.000001	"	++++	+
0.0000001	"	0	0
0.001	1:250,000	++++	+
0.0001	"	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
0.01	1:2,500,000	++++	+
0.001	"	0	0
0.0001	"	0	0
0.00001	"	0	0
0.000001	"	0	0

TABLE II—*Concluded.*

Pneumococci Type II. Amount of standard suspension.	Soluble specific substance. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
	Type III.		
0.01	1:250	++++	+
0.001	"	0	0
0.0001	"	0	0
0.00001	"	0	0
0.000001	"	0	0
Controls without leucocytes.			
0.0000001	0		+
0.0000001	Type II S 1:250		+
0.0000001	Type III S 1:250		+
Plating.	No. of colonies.		
0.0000001	105		
0.0000001	102		

again indicated by the failure of Type III soluble substance to produce any effect even in high concentration.

To test further the specificity of this reaction, the effect of Type II and Type III soluble specific substances on Pneumococcus Type III was studied.

Experiment 3.—(Table III.) Cat serum and cat leucocytes employed. Series of tubes were set up with Pneumococcus Type III and Types III and II soluble substances respectively.

It is seen in Table III that with quantities of pneumococci representing less than 0.0001 cc. of the standard suspension, growth inhibition occurred in the cat serum-leucocyte mixtures. But the presence of soluble substance of the homologous type in a dilution of 1:25,000 was able to cause the growth of even a very small number of pneumococci. The Type II soluble substance had no such action.

Experiments were next carried out with broth filtrates from young pneumococcus cultures with the purpose of determining whether the action of the highly purified soluble substance just described is identi-

TABLE III.

Cat serum 0.2 cc. + cat leucocyte suspension 0.1 cc. + soluble specific substance 0.1 cc. + pneumococcus suspension 0.1 cc.

Pneumococci Type III. Amount of standard suspension.	Soluble specific substance. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
Type III.			
cc.			
0.0001		++++	+
0.00001		0	0
0.000001		0	0
0.0000001		0	0
0.000001	1:2,500	++++	+
0.0000001	"	++++	+
0.000001	1:10,000, 1:25,000	++++	+
0.0000001	respectively.	++++	+
0.0001	1:250,000	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
Type II.			
0.0001	1:2,500	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
Controls without leucocytes.			
0.0000001	0		+
0.0000001	Type III S 1:2,500		+
0.0000001	Type II S 1:2,500		+
Plating.	No. of colonies.		
0.0000001	139		
0.0000001	137		

cal with that of the substance originally found in the broth culture of pneumococcus.

Experiment 4.—(Table IV.) Rabbit serum and leucocytes used. Broth filtrates

TABLE IV.

Rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + broth filtrate 0.1 cc. + pneumococcus suspension 0.1 cc.

Pneumococci Type II. Amount of standard suspension.	Broth filtrate. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
	Type II.		
cc.			
0.001		++++	+
0.0001		0	0
0.00001		0	0
0.000001		0	0
0.000001	1:5, 1:25, 1:50,	++++	+
0.0000001	1:100, 1:200 respectively.	++++	+
0.00001	1:400	++++	+
0.000001	"	++++	+
0.0000001	"	0	0
0.001	1:800	++++	+
0.0001	"	0	0
0.00001	"	0	0
0.000001	"	0	0
	Type III.		
0.001	1:5	++++	+
0.0001	"	0	0
0.00001	"	0	0
0.000001	"	0	0
Control series with:	Plain broth.		
0.001	1:5	++++	+
0.0001	"	0	0
0.00001	"	0	0
0.000001	"	0	0
Controls without leucocytes.			
0.0000001	0		+
0.0000001	Type II B.F.* 1:5		+
0.0000001	Type III B.F. 1:5		+
0.0000001	Plain broth 1:5		+

* B.F. = Broth filtrate.

from Type II and Type III pneumococci employed with a Pneumococcus Type II. As a further control on the broth filtrates, a series of tubes was also set up with plain meat infusion broth.

TABLE V.

Cat serum 0.2 cc. + cat leucocyte suspension 0.1 cc. + broth filtrate 0.1 cc. + pneumococcus suspension 0.1 cc.

Pneumococci Type III. Amount of standard suspension.	Broth filtrate. Final dilution.	At 72 hrs.	
		Color change.	Pneumococci in stained film.
Type III.			
cc.			
0.0001		++++	+
0.00001		0	0
0.000001		0	0
0.0000001		0	0
0.000001	1:5	++++	+
0.0000001	"	++++	+
0.000001	1:50, 1:100, 1:200,	++++	+
0.0000001	1:400 respectively.	++++	+
Type II.			
0.0001	1:5	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
Control series with:	Plain broth.		
0.0001	1:5	++++	+
0.00001	"	0	0
0.000001	"	0	0
0.0000001	"	0	0
Controls without leucocytes.			
0.0000001	0		+
0.0000001	Type III B. F. 1:5		+
0.0000001	Type II B. F. 1:5		+
0.0000001	Plain broth 1:5		+

It was found that the addition of a small quantity of the Type II broth filtrate to normal rabbit serum-leucocyte mixtures acted in very

much the same way as did the Type II soluble substance in Experiment 1 (Table IV). The action of this broth filtrate could still be seen in a dilution as high as 1:400. On the other hand, Type III broth filtrate, even in a concentration of as high as 1:5, failed to show such action.

The fact that the Type III broth filtrate used gave a positive precipitin reaction in a dilution as high as 1:40 made it seem evident that the failure of this filtrate to promote growth of the Type II pneumococcus was not due simply to a lack of the Type III active substance. But in order to test directly the potency of the Type III broth filtrate, the following experiment was performed.

Experiment 5.—(Table V.) Cat serum and cat leucocytes again employed. Pneumococcus Type III was used as the test organism with the Types III and II broth filtrates respectively.

It is seen from Table V that the Type III broth filtrate was highly active. A dilution as high as 1:400 was still sufficient to enable the growth to occur of even the smallest number of pneumococci in the serum-leucocyte mixtures when a Type III pneumococcus was used as the test organism. How much further this broth filtrate could be diluted before its action would have disappeared was not determined. The specificity of the broth filtrate soluble substance was again well shown by the inability of the Type II broth filtrate to alter the growth condition of the Type III pneumococcus in the cat serum-leucocyte mixtures.

DISCUSSION.

The experiments here reported show clearly that the soluble specific substance of pneumococcus exerts a definite action on the growth of pneumococci in the serum-leucocyte mixtures. Its presence markedly alters conditions in the mixture so that even a very small number of avirulent pneumococci are enabled to grow in the serum-leucocyte mixtures of a highly resistant animal which normally possess the power to destroy such pneumococci in relatively large numbers. It was found in previous studies that the ability of pneumococcus to grow in serum-leucocyte mixtures depends on its virulence for the species. The absence of growth inhibition was found invariably to accompany a high degree of virulence. Thus the effect of the soluble substance

in the serum-leucocyte mixtures is to confer on the avirulent pneumococcus the growth capacity of a virulent organism.

The mechanism of the reaction is as yet not well understood. Preliminary experiments have definitely shown that the leucocytes are not injured by the soluble substance. From the studies of Rosenow (3), it would seem likely that the soluble substance neutralizes the opsonin in the serum, as a result of which the organisms resist phagocytosis and growth occurs. The specific action of the soluble substance described in this communication, however, needs further explanation. Rosenow (3) showed that human serum contains specific opsonins for pneumococci, staphylococci, streptococci, and tubercle bacilli, and Bull and McKee (10) demonstrated the existence of separate protective substances in normal chicken serum for each type of pneumococcus. May it not be possible that rabbit and cat serum also possess separate normal opsonins specific for each serological type of pneumococcus of low virulence for the species?

SUMMARY.

By means of a method previously described for demonstrating the growth-inhibitory and pneumococcidal power of normal serum-leucocyte mixtures, a study was made to determine the action of pneumococcus soluble substance on the growth of strains of pneumococci avirulent for rabbits and cats. It was found that the presence of a very small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixture so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which possess the power to destroy ordinarily such pneumococci in relatively large numbers. The action of the soluble substance was shown to be highly specific to type. A Type II substance assisted the growth of only Pneumococcus Type II, likewise a Type III substance, the growth of Pneumococcus Type III only.

Experiments involving broth filtrates of young pneumococcus cultures in place of the purified soluble substances gave similar results, thereby establishing further the identity of the purified substance with the precipitable substance originally described in the filtrates of pneumococcus cultures.

The effect of the soluble specific substance of pneumococcus in the serum-leucocyte mixtures is, therefore, to confer on the avirulent pneumococcus of the homologous type the growth capacity of a virulent organism.

The mechanism of the reaction is briefly discussed.

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SOME CONDITIONS OF THE REPRODUCTION IN VITRO OF THE ROUS VIRUS.

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Although the Rous virus, so called, of chicken tumor multiplies readily *in vitro*,[1] we cannot consider that it has been positively shown to be an ultramicroscopic organism. It may be conceived to be a self-reproducing enzyme, or a minute bacterium; a principle foreign to the host and using for its growth some of the tissue constituents; or a substance generated and reproduced by the tissues themselves. Several years ago, Twort[2] expressed the opinion that a substance analogous to the autolyzing substance he discovered in his cultures of micrococci might be responsible for malignant tumors. When the Rous sarcoma agent or virus was found to grow in a culture medium containing living leucocytes, it became obvious that the factor responsible for its reproduction was not the medium but the cells that proliferate in it. Although these cells were not always modified in their appearance, they generally showed certain morphological changes which were probably caused by the presence of the virus at the surface of or inside their body.[3] So far, in these experiments as in the more recent ones of Gye,[4] the agent has never been found to multiply in a medium that contains no fresh tissue. In the studies reported in the present paper, an attempt was made to ascertain the relations between the reproduction *in vitro* of the Rous virus and the quantity, the activity, and the nature of the cells contained in the medium.

Technique for Detecting the Presence of Rous Virus in a Fluid.

The presence of Rous virus in the fluid of a culture could be easily detected by inoculating chickens with this fluid, provided these animals

[1] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.
[2] Twort, F. W., *Lancet*, 1915, ii, 1241.
[3] Carrel, A., *Compt. rend. Soc. biol.*, 1924, xci, 1069; 1925, xcii, 584.
[4] Gye, W. E., *Lancet*, 1925, ii, 109.

were all equally sensitive to the sarcoma agent. But such is not the case. When a number of chickens are inoculated with a diluted extract of Rous sarcoma, some develop a tumor, while others do not. There are marked variations in the individual resistance of the fowls.

The inoculation of a given amount of diluted extract in different parts of the body of a single chicken does not bring about the simultaneous development of tumors of the same size. The manner in which the fluid is injected and diffuses through the tissues, the site of the inoculation, the local condition of the tissues, etc., have a marked influence on the rate of appearance of the tumor and on its growth. The diffusion of the fluid often renders impossible the comparison of several inoculations in the same animal. In addition, it is not possible to compare different fluids by inoculating them into different chickens, on account of the variations in the individual susceptibility of the animals. A technique had to be developed that would permit the inoculation under identical conditions of equal amounts of fluids, and the testing on the same animal of several extracts.

In this study, sarcomas were used that had grown for about a week in chickens of approximately the same age and breed. The tissues were pulped in a Latapie apparatus. The pulp was mixed with its volume of Tyrode solution and placed for 1 hour in the refrigerator at a temperature of about 4°C. The fluid obtained by centrifugation of the suspension was used as "normal extract." This normal extract was diluted with Tyrode solution and dilutions from 1 in 10 to 1 in 50,000 were prepared. Small discs of woolen cloth 5 mm. in diameter were placed for 1 hour in the fluid to be tested. Each disc absorbed approximately 0.04 cc. of the fluid. Four or five discs were grafted on each side of the sternum in small pockets made in the subcutaneous connective tissue.

When the details of the technique were carefully carried out, the inoculation of a chicken with eight or ten discs soaked in fluids containing progressively decreased concentrations of Rous virus often gave rise in about 3 weeks to eight or ten small tumors of regularly decreasing size. It has, then, become possible to test the susceptibility of an animal by inoculating it with various dilutions of Rous virus, and observing the lowest dilution that brings about the appearance of a tumor. When the discs were soaked in from 1 per 10 to 1 per 100 extracts, tumors developed in practically every chicken. Almost all the animals were susceptible to Rous virus at these concentrations.

When more diluted solutions were used, great individual differences were found. Chickens grafted with discs containing Rous virus in concentrations from 1 per 1,000 to 1 per 50,000 showed wide variations in resistance. Tumors were often brought about by the solutions 1 per 1,000 and 1 per 10,000, while they occurred less frequently at concentrations of 1 per 20,000 and 1 per 30,000, seldom at 1 per 40,000, and practically never at 1 per 50,000. The susceptibility to Rous virus is generally a permanent characteristic of an animal. When the tumors were extirpated and no generalization of the disease occurred, the animal could be inoculated again after a few months, and usually showed the same degree of susceptibility as before. The resistant animal remained resistant over long periods of time. But an animal that was resistant on account of a temporary cause, such as an accidental illness, might become susceptible when it had recovered its health.

Since Rous virus does not produce a tumor even in a susceptible fowl when inoculated at concentrations equal to or higher than 1 per 50,000, or in more resistant animals at concentrations from 1 per 10,000 to 40,000, the negative result of an inoculation may indicate either that the virus is lacking, or that it is present in an amount smaller than the minimum concentration required for the growth of a tumor by the inoculated animal. These variations of the individual resistance do not allow a comparison of the virulence of fluids when injected into different chickens. But the presence and the amount of the diluted sarcoma principle can be ascertained when the fluids, together with a proper control, are injected into the same animal. Although the method is still far from being precise, it makes it possible to ascertain whether one culture contains more virus than another, and whether the amount is large or small.

The Reproduction in Vitro of the Filter-Passing Principle or Virus in the Presence of Living Cells.

The well known experiments of Rous have demonstrated that the sarcoma agent is very fragile, and that its activity disappears in less than 24 hours when it is in suspension in a fluid medium such as saline solution, bouillon, or serum. These findings have been confirmed many times by other workers. In the course of experiments made in

September, 1924, the addition of a fragment of muscle or of some leucocytes to a suspension of Rous virus, which lost its activity at incubator temperature in less than 24 hours, was found to increase the duration of the activity to at least 48 hours. At the same time, it appeared that, in a medium containing active leucocytes the virus not only survived but seemed even to multiply. Although these experiments have already been mentioned in a previous paper,[1] the details of the technique employed and results achieved are only now described.

Leucocytes were obtained from chicken blood and embedded in a film of plasma. The film was cut into rectangular fragments of approximately equal size. Three or four fragments were placed in a flask containing a medium composed of 0.5 plasma, 1.5 Tyrode solution, and 0.5 embryonic tissue juice. On the surface of the solid medium was injected 1 cc. of a mixture of serum, Tyrode solution, and tissue juice. The culture was inoculated with filtered extract of Rous sarcoma which was added directly to the fluid medium, or the leucocytic films were placed in the extract for some time before the preparation of the culture. In one of the earlier experiments, the culture was inoculated with a fragment of sarcoma which had been killed by drying *in vacuo* over sulfuric acid. Every 2 or 3 days, the fluid medium was removed and replaced by fresh fluid, after the coagulum had been washed with Tyrode solution. From time to time, tests were made to ascertain whether the leucocytes or the medium had undergone any transformation under the influence of the virus. This was done by inoculating chickens with a fragment of the coagulum or part of the fluid medium.

A first series of twenty-two experiments was made in September and October, 1924 (Table I).

After the cultures had been incubated from 4 to 30 days, fragments of the coagulum or discs soaked in the supernatant fluid of the cultures were inoculated into chickens. In twenty-four inoculations, seven gave negative and seventeen positive results. The seventeen tumors were produced in six cases by inoculation of a fragment of coagulum and in eleven cases by inoculation of the fluid. The six tumors produced by inoculation of a fragment of coagulum developed in an average time of 15 days, and the eleven tumors due to the graft of discs saturated with 0.04 cc. of culture fluid in an average time of 16.8 days. As some of the tumors were already large when observed for the first time, they really appeared earlier than is indicated in the table. The seventeen cultures that brought about a tumor upon

inoculation showed in six cases no change in the appearance of the coagulum or of the cells. In the other cases, clumps of dead cells, digestion of the coagulum, and fibroblast forms were observed. All the chickens ultimately died from the tumors.

Many experiments of the same type have since been made. The technique was modified slightly. The filtered extract was inoculated into the solid medium at the time of the preparation of the cultures. Embryonic pulp or fragments of spleen were often used instead of leucocytic film. The cultures were inoculated not only with the Rous virus, but also with the filtered extracts of tar, arsenic, and indole sarcomas. The filter-passing agent of the tar and arsenic sarcomas remained active in the presence of spleen, embryonic pulp, and leucocytes, as did Rous virus. So far, the filtered agent of the indole tumor has not kept its activity *in vitro*, although its inoculation always has induced large sarcomas in chickens. The cultivation *in vitro* of the agent of Rous sarcoma and the sarcomas of chemical origin is used as a routine procedure for keeping on hand a constant supply of active virus. When some fragments of leucocytic film or of spleen are added to the medium from time to time, and the cultures are transferred into new flasks every 2 or 3 weeks, the filter-passing agent maintains its virulence. *Such cultures that have been kept for over 2 months produce at least 1 cc. of highly virulent fluid every day.* It is obvious that the sarcoma agent not only remains in the flasks, but also multiplies very actively.

Rôle of the Medium and the Tissues in the Reproduction of the Rous Virus.

While the foregoing experiments show that the Rous agent multiplies in a medium containing living cells, they do not indicate what rôle belongs to the cells and the medium respectively. Often, the cells are profoundly modified in their morphology and functions, changes that we have described as a malignant transformation.[1] But in some cases, the Rous virus grows in the medium in the presence of monocytes that appear quite normal. The same phenomenon has been observed to take place in pure cultures of macrophages obtained from Rous sarcoma, which do not seem to differ from cultures of blood monocytes. It is probable that Rous agent reproduces itself at the surface of or inside the cells. But it is also conceivable that it multiplies in

TABLE I.

Increase of the Rous Virus in Vitro in the Presence of Leucocytes.

Experiment No.	Culture No.	Date of preparation of cultures.	Period of incubation of cultures.	Changes in medium and cells.	Date of inoculation into chickens.	Inoculation No.	Mode of inoculation.	Results of inoculation.
		1924	*days*		*1924*			
1	1780 A	Sept. 20	4	No change.	Sept. 24	304 B	Fragments of coagulum.	No tumor.
2	1795 A	" 24	2	" "	" 26	307 B	" " "	" "
3	1800 A	" 25	4	" "	" 29	310 B	" " "	" "
4	1754 A	" 16, 18	20	Dead cells; no digestion.	Oct. 6	323 B	" " "	Large tumor, Oct. 22.
5	1752 A	" 16–18	20	Fibroblastic forms; no digestion.	" 6	324 B	" " "	" " " 15.
6	1774 A	" 19	19	No change.	" 8	327 B	" " "	Tumor, Oct. 25.
7	7247 D	" 25	23	" "	" 18	342 B	" " "	Large tumor, Oct. 29.
8	1887 A	Oct. 16	4	" "	" 20	343 B	Fluid.	" " Nov. 12.
9	7217 D	" 3	17	Dead cells; coagulum digested.	" 20	344 B	"	" " " 1.
10	1887 A	" 16	4	No change.	" 20	344 B	"	Tumor, Nov. 11.
11	7250 D	Sept. 27	23	Dead cells; coagulum digested.	" 20	345 B	"	" " 1.
12	564 C	Oct. 16	4	Dead cells; no digestion.	" 20	345 B	"	" " 11.
						346 B	"	Small tumor, Nov. 1.
						347 B	"	Large " " 8.
13	7250 D	Sept. 27	23	Dead cells; coagulum digested.	" 20	347 B	"	Tumor, Nov. 8.

14	1887 A	Oct. 16	4	No change.	Oct. 20	348 B	Fluid.	Large tumor, Nov. 8.
15	7250 B	Sept. 27	25	Dead cells; fibroblasts digested.	" 22	349 B	"	Tumor, Nov. 1.
16	7217 B	Oct. 3	19	" "	" 22	349 B	"	No tumor.
17	7217 B	" 3	21	" "	" 24	353 B	Fragments of coagulum.	Large tumor, Nov. 8.
18	564 C	" 16	8	No change.	" 24	353 B	" " "	Tumor, Nov. 15.
19	7281 D	" 14	19	Dead cells; coagulum digested.	Nov. 3	371 B	Fluid.	" " 18.
20	625 C	" 31	13	No change.	" 13	390 B	"	No tumor.
21	1887 A	" 16	28	" "	" 14	392 B	"	" "
22	1887 A	" 16	32	" "	" 18	400 B	"	" "

TABLE II.

Tests of the Complement Effect of a Medium Containing Living Tissues on a Medium without Tissues but Inoculated with Rous Virus.

Experiment No.	Culture No.	Nature of tissue.	Quantity of virus.	Date of preparation of cultures.	Date of inoculation into chickens.	Inoculation No.	Results of inoculation.
			cc.	*1925*	*1925*		
1	8268-1 D	Spleen, 5 fragments.	1	Nov. 17	Nov. 19	1085 B	Tumor.
					" 20	1090 B	"
					" 23	1096 B	No tumor, bacterial infection.
					" 28	2008 B	No tumor.
	8268-2 D	No tissue.	1	" 17			
	8268-3 D	Spleen, 5 fragments.	0	" 17			
	8268-2 and 3 D				" 19	1085 B	" "
					" 20	1090 B	" "
					" 23	1096 B	" "
					" 28	2008 B	" "
2	2870-1 A	Leucocytes, 5 fragments.	0.5	" 17	" 20	1090 B	" "
					" 23	1095 B	Tumor.
					" 25	2007 B	"
					" 30	2023 B	"
					Dec. 2	2029 B	"
					" 4	2036 B	No tumor.
	2870-2 A	No tissue.	0.5	" 17	Nov. 30	2023 B	" "
					Dec. 2	2029 B	" "
					" 4	2036 B	" "
	2870-3 A	Leucocytes, 5 fragments.	0	" 17	Nov. 30	2023 B	" "
					Dec. 2	2029 B	" "
					" 4	2036 B	" "

2	2870-2 and 3 A				Nov. 20	1090 B	No tumor.
					" 23	1095 B	" "
					" 25	2007 B	" "
					" 30	2023 B	" "
					Dec. 2	2029 B	" "
					" 4	2036 B	" "
3	2871-1 A	Embryo pulp, 9 drops.	0.5	Nov. 17	Nov. 20	1090 B	" "
					" 23	1095 B	" "
					" 25	2006 B	Tumor.
					" 27	2015 B	No tumor.
	2871-2 A	No tissue.	0.5	" 17			
	2871-3 A	Embryo pulp, 9 drops.	0	" 17			
	2871-2 and 3 A				" 20	1090 B	" "
					" 23	1095 B	" "
					" 25	2006 B	" "
					" 27	2015 B	" "
4	8311-1 D	Leucocytes, 8 fragments	1	" 27	Dec. 1	2026 B	Tumor.
					" 1	2027 B	No tumor.
					" 3	2032 B	Tumor.
					" 8	2042 B	"
	8311-2 D	No tissue.	1	" 27	Dec. 1	2026 B	No tumor.
					" 1	2027 B	" "
					" 3	2032 B	" "
					" 8	2042 B	" "
	8311-3 D	Leucocytes, 8 fragments.	0	" 27	" 1	2026 B	" "
					" 1	2027 B	" "
					" 3	2032 B	" "
					" 8	2042 B	" "
	8311-2 and 3 D				" 1	2026 B	" "
					" 1	2027 B	" "
					" 3	2032 B	" "
					" 8	2042 B	" "

the medium in an inactive form, as Gye has supposed, and that it receives from the growing cells a complementary or activating substance. According to this hypothesis, the active virus derived from our cultures might be considered as composed of two parts: one inactive and multiplying in the medium, and the other activating and produced by the tissues. In order to test the value of this hypothesis, experiments of the following type were made.

In three flasks, equal amounts of medium were introduced. In addition, Flask 1 received some virus and embryonic pulp; Flask 2, some virus and no embryonic pulp; and Flask 3, embryonic pulp alone. From time to time, a small amount of the fluid was withdrawn from the cultures. Three discs were soaked in the three fluids, and a fourth disc in a mixture of the fluids from the second and third flasks. The four discs were grafted under the skin of the thorax of a chicken. The nature of the tissues, the amounts of tissues and virus used, the duration of the incubation of the cultures, and the dates of their inoculation and of the appearance of the tumors are recorded in Table II.

The fluid of the flasks was inoculated into chickens four, five, or six times, from 2 to 18 days after the preparation of the cultures. The results were uniform. A tumor was produced by the fluid of the first flask, and no tumor occurred when the fluids of the second and third flasks were inoculated separately or together. Since the injection of a mixture of the fluid of the second and third flasks does not produce a tumor, the rôle of the cells contained in the first flask does not consist in supplying to a virus multiplying within the medium in an inactive state a substance that would activate it. The lack of activity of the fluid of the second flask must be attributed to the disappearance of the virus, or to its reproduction in a concentration less than that of 1 in 20,000 to 1 in 50,000 which is required for the development of a sarcoma. Thus, it appears that the rôle of the cells is to promote directly the growth of the active virus. But this fact does not mean that living cells may not set free substances capable of increasing its activity. In other experiments, it was found that the addition of embryonic juice to Rous virus rendered inactive by its high dilution, might enable this inactive virus to produce a tumor.

Relation between the Quantity of Tissues Contained in the Medium and the Reproduction of the Virus.

In two flasks, equal amounts of medium and sarcoma extracts were placed. Into the first flask a drop of embryonic pulp was introduced, and into the second flask, 9 drops. Identical experiments were made with fragments of spleen and of leucocytic film. After from 2 to 16 days incubation, the fluid from the cultures was removed, and discs that had been soaked in it were inoculated into chickens. The fluid from the flasks containing only 1 drop of embryonic pulp, or a fragment of spleen or leucocytic film, generally did not induce a tumor, while a sarcoma developed almost always at the site of inoculation of the fluid from the flasks containing the larger amount of tissue. The details of the experiments are described in Table III.

Nine experiments were made, three with embryonic pulp, three with leucocytes, and three with spleen. The cultures were inoculated with filtered or unfiltered extracts of tar sarcoma and arsenic sarcoma, in amounts varying from 1.5 cc. to 0.5 cc. The fluids of the cultures were removed every 2 or 3 days, the cultures were washed, and fresh fluid was added. Previous to the inoculation, the cultures were incubated ten times for less than 5 days, five times from 5 to 10 days, and eight times from 10 to 16 days. Culture 2872 was kept after the end of the experiment described in the table and, after 2 months incubation, still produced an active virus. The fluid from the cultures containing the larger amount of tissue was inoculated twenty-four times and produced a tumor eighteen times, while the fluid from the flasks containing the smaller amount was inoculated twenty-two times and produced a tumor only thrice. In these cases, the cultures from which the fluid was taken contained leucocytes or spleen. The minimum amount of spleen necessary for producing in 3,000 c.mm. of medium enough virus to develop a tumor upon inoculation into a chicken appeared to be approximately 1 c.mm. of spleen. When the amount of tissue was from five to nine times the minimum used, the production of a tumor by the fluid of the culture was frequent.

TABLE III.

Relation between the Increase in Quantity of the Sarcoma Agent and the Quantity of Tissue Contained in the Medium.

Experiment No.	Culture No.	Nature and quantity of tissue.	Nature and quantity of virus.	Date of preparation of cultures.	Changes in medium and tissues.	Date of inoculation into chickens.	Inoculation No.	Results of inoculation.
				1925		*1925*		
1	2719-1 A	No tissue.	0.5 cc. filtered extract of arsenic sarcoma.	Sept. 24	No change.	Sept. 28	927 B	No tumor.
						Oct. 6	946 B	" "
	2719-2 A	Spleen, 4 fragments.	" "	" 24	Medium digested, dead cells.	Sept. 28	927 B	" "
						Oct. 6	946 B	Tumor.
2	8181-1 D	Embryo pulp, 1 drop.	0.5 cc. filtered extract of tar sarcoma.	Oct. 27		Nov. 2	1035 B	No tumor.
	8181-2 D	Embryo pulp, 6 drops.	" "	" 27		" 2	1035 B	Tumor.
3	8223-1 D	Embryo pulp, 2 drops.	1.5 cc. unfiltered extract of tar sarcoma.	" 27		" 6	1047 B	No tumor.
						" 7	1048 B	" "
						" 9	1051 B	" "
	8223-2 D	Embryo pulp, 9 drops.	" "	" 27		" 6	1047 B	Tumor.
						" 7	1048 B	"
						" 9	1051 B	"

4	2807-1 A	Embryo pulp, 1 drop.	0.5 cc. filtered extract of tar sarcoma.	Oct. 26		Oct. 29	1028 B	No tumor
	2807-2 A	Embryo pulp, 9 drops.	" "	" 26		" 29	1028 B	Tumor.
5	8267-1 D	Spleen, 1 fragment.	1 cc. filtered extract of tar sarcoma.	Nov. 17	Medium digested, dead cells.	Nov. 19	1086 B	No tumor.
						" 23	1097 B	" "
						" 25	2008 B	" "
	8267-2 D	Spleen, 5 fragments.	" "	" 17	" "	" 19	1086 B	" "
						" 23	1097 B	" "
						" 25	2008 B	Tumor.
						Dec. 1	2027 B	"
						" 1	2028 B	"
6	8268-1 D	No tissue.	" "	" 17	No change.	Nov. 19	1085 B	No tumor.
	8268-2 D	Spleen, 5 fragments.	" "	" 17	Medium digested, dead cells.	" 19	1085 B	Tumor.
7	8272-1 D	Leucocytes, 1 fragment.	" "	" 17	No digestion, many dead cells.	" 19	1085 B	"
						" 20	1089 B	No tumor.
	8272-2 D	Leucocytes, 5 fragments.	" "	" 17	" "	" 19	1085 B	Tumor.
						" 20	1089 B	No tumor.
8	8282-1 D	Leucocytes, 1 fragment.	1.5 cc. unfiltered extract of tar sarcoma.	Nov. 19	No change.	Nov. 21	1092 B	No tumor.
	8282-2 D	Leucocytes, 8 fragments.	" "	" 19	" "	" 21	1092 B	Tumor.

TABLE III—*Concluded.*

Experiment No.	Culture No.	Nature and quantity of tissue.	Nature and quantity of virus.	Date of preparation of cultures.	Changes in medium and tissues.	Date of inoculation into chickens.	Inoculation No.	Results of inoculation.
				1925		*1925*		
9	2872-1 A	Leucocytes, 1 fragment.	0.5 cc. filtered extract of tar sarcoma.	Nov. 17	No change.	Nov. 19	1085 B	Small tumor.
						" 19	1087 B	No tumor.
						" 20	1089 B	" "
						" 23	1095 B	" "
						" 25	2007 B	Small tumor.
						" 30	2023 B	No tumor.
						Dec. 1	2026 B	" "
						" 3	2032 B	" "
	2872-2 A	Leucocytes, 9 fragments.	" "	" 17	Medium digested, dead cells.	Nov. 19	1085 B	Large tumor.
						" 19	1087 B	No tumor.
						" 20	1089 B	" "
						" 23	1095 B	Tumor.
						" 25	2007 B	Large tumor.
						" 30	2023 B	Tumor.
						Dec. 1	2026 B	"
						" 3	2032 B	"

TABLE IV.

Relation between the Nature of the Cells Contained in the Medium and the Increase in Quantity of the Sarcoma Agent.

Experiment No.	Culture No.	Nature of tissue.	Nature and quantity of virus.	Date of preparation of cultures.	Changes in tissues.	Date of inoculation into chickens.	Inoculation No.	Results of inoculation.
				1925		*1925*		
1	8299-1 and 2 D	Blood leucocytes.	1 cc. Rous sarcoma extract.	Nov. 24	No change.	Nov. 30	2023 B	Tumor.
						Dec. 7	2040 B	"
	8299-3 and 4 D	Fibroblasts, 13 yr. old strain.	" "	" 24	" "	Nov. 30	2023 B	No tumor.
						Dec. 7	2040 B	" "
2	8287-1 and 2 D	Blood leucocytes.	1 cc. fluid from culture of tar sarcoma.	" 20	" "	Nov. 21	1091 B	Tumor.
						" 23	1098 B	No tumor.
	8287-3 and 4 D	Fibroblasts, 13 yr. old strain.	" "	" 20	" "	" 21	1091 B	" "
						" 23	1098 B	" "
3	8336 D	" "	1 cc. Rous sarcoma extract.	Dec. 2	" "	Dec. 7	2040 B	" "
4	8367 D	" "	0.5 cc. fluid from culture of Rous sarcoma.	" 11	Appearance of macrophages.	Dec. 17	2084 B	" "
						" 22	2096 B	" "
5	8376 D	" "	1 cc. Rous sarcoma extract.	" 12	No change.	" 17	2096 B	" "
						" 23	2126 B	" "
					Digestion.	" 30	2096 B	" "

Relation between the Nature of the Cells Contained in the Medium and the Reproduction of Rous Virus.

When pure cultures of fibroblasts obtained from a 13 year old strain were placed in a medium containing Rous virus, and after a few weeks were grafted into chickens, no tumors developed.[5] By contrast, after cultures of leucocytes had been inoculated with Rous agent, their supernatant fluid led to the development of a sarcoma in chickens, even when the monocytes had not undergone malignant transformation.[6] It seemed as if the sarcoma agent reproduced readily in the presence of monocytes and did not in the presence of fibroblasts. But in these early experiments, the mass of the tissues was generally smaller in the cultures of fibroblasts than in those of monocytes. The negative results of the graft of the fibroblast cultures could be ascribed, not to the absence of Rous virus, but to its low concentration. The purpose of the following experiments was merely to ascertain whether fibroblasts, in larger quantities than previously used, would favor survival and production of the virus (Table IV).

In Experiments 1 and 2, four fragments of leucocytes and four fragments of fibroblast tissue from the 13 year old strain were placed in flasks containing either extract of sarcoma at 1 in 10 dilution, or some fluid from cultures of the Rous or tar sarcomas. The fluid of the leucocytic cultures was injected into chickens from 1 to 13 days after the preparation of the flasks. Three times in four, the inoculations produced a tumor. The fluid of the cultures of fibroblasts, which contained approximately the same volume of tissue as the cultures of leucocytes, was injected into chickens from 1 to 18 days after the preparation of the flask. Eight injections were made, and no tumors occurred. These experiments showed that the virus rapidly disappears from the fluid of the cultures of fibroblasts, while it remains present in the cultures of leucocytes. Although the fibroblasts did not set free in the culture medium any virus at a concentration capable of producing a tumor upon inoculation into chickens, they were infected as was shown subsequently. On January 12 and 16, that is approximately 1 month after the preparation of Cultures 8367

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1925, xcii, 477.
[6] Carrel, A., *Compt. rend. Soc. biol.*, 1924, xc, 1380.

and 8376, the fibroblasts themselves, instead of the fluid, were grafted into chickens, and sarcomas developed. This experiment demonstrated that in the presence of equal volumes of fibroblasts and leucocytes, the Rous virus kept its full activity in the fluid of the leucocytic cultures, while it disappeared promptly from the fluid of the cultures of fibroblasts. But, after its disappearance from the fluid, the virus still remained present for several weeks within the colonies of fibroblasts themselves. Leucocytes and fibroblasts acted on the virus in a markedly different manner.

Relation between Cell Activity and the Reproduction of the Virus.

The purpose of the experiment was to determine whether the Rous agent survives or multiplies in tissues that do not proliferate (Table V).

The tissues used in these experiments were embryo pulp, leucocytes, and spleen. The virulence of the fluid of a culture of normal tissues inoculated with virus was compared with that of a culture of the same tissues after they had been frozen or kept in an anaerobic condition for 24 or 48 hours. In the tissues that had been frozen, only a few cells were still alive after a few days incubation. No living cells were seen in the cultures that had been kept under anaerobic conditions for 24 or 48 hours. In Experiment 1, after 28 hours incubation, the inoculation of fluid of the culture containing normal embryo pulp determined a tumor, while that of the culture of frozen tissue remained negative. In Experiment 2, after 3 days incubation, the fluid of the control produced a large tumor, and that of the culture of frozen tissue, which did not contain any active cells, determined a small tumor. Both control and experiment cultures contained the same amount of tissue. In Experiments 3, 4, 5, and 6, embryo pulp, spleen, and leucocytes, inoculated with virus, were cultivated in the usual manner after having been kept 24 or 48 hours in an anaerobic jar. Seven inoculations of the fluids of the controls produced a tumor six times, while the same number of inoculations of the tissue deprived of oxygen produced only two small tumors. The cultures had been incubated for 6 days and no living cells could be seen. This last experiment indicates that in the presence of dead cells the virus can still survive, but that cell proliferation seems to be necessary for its abundant reproduction in an active state.

TABLE V.

Relation between Cell Activity and Increase of the Sarcoma Agent.

Experiment No.	Culture No.	Nature of tissues and medium.	Tissues, normal or frozen.	Aerobic or anaerobic.	Date of preparation of cultures.	Changes in medium and tissues.	Date of inoculation into chickens.	Inoculation No.	Results of inoculation.
					1925		*1925*		
1	8177-1 D	Embryo pulp, chicken plasma.	Normal.	Aerobic.	Oct. 26	Large growth.	Oct. 28	1021 B	Tumor, Nov. 11.
	8177-2 D	" "	Frozen.	"	" 26	Almost no growth.	" 28	1021 B	No tumor.
2	8278-1 D	" "	Normal.	"	Nov. 18	Large growth.	Nov. 20	1089 B	Tumor, Dec. 11.
						" "	" 21	1091 B	Large tumor, Dec. 16.
						" "	" 23	1098 B	No tumor.
						" "	" 24	2000 B	" "
	8278-2 D	" "	Frozen.	"	" 18	No growth.	" 20	1089 B	" "
						" "	" 21	1091 B	Small tumor, Dec. 16.
						" "	" 23	1098 B	No tumor.
						" "	" 24	2000 B	" "
3	8220-1 D	Embryo pulp, chicken serum.	Normal.	"	" 5	No visible change.	" 11	1063 B	Tumor, Dec. 7.
	8220-2 D	" "	"	Anaerobic, 48 hrs.	" 5	" "	" 11	1063 B	Small tumor, Dec. 7.

4	8220-3 D	Embryo pulp, rabbit serum	Normal.	Aerobic.	Nov. 5	No visible change.	Nov. 11	1063 B	Large tumor, Dec. 7.
	8220-4 D	" "	"	Anaerobic, 48 hrs.	" 5	" "	" 11	1063 B	No tumor.
5	8271-1 D	Leucocytes, 6 fragments.	"	Aerobic.	" 17	Large growth, medium digested.	" 23	1097 B	Tumor, Dec. 7.
	8271-2 D	" "	"	Anaerobic, 24 hrs.	" 17	No growth, medium not digested.	" 23	1097 B	Small tumor, Dec. 23.
6	8267-1 D	Spleen, 6 fragments.	"	Aerobic.	" 17	Large growth, medium digested.	" 23	1097 B	No tumor.
							" 28	2008 B	Tumor, Dec. 12.
							Dec. 1	2027 B	Large tumor, Dec. 15.
							" 1	2028 B	Tumor, Dec. 15.
	8267-2 D	" "	"	Anaerobic, 24 hrs.	" 17	No growth, medium not digested.	Nov. 23	1097 B	No tumor.
							" 28	2008 B	" "
							Dec. 1	2027 B	" "
							" 1	2028 B	" "

SUMMARY AND CONCLUSIONS.

A method has been developed by which the susceptibility of chickens to Rous virus can be tested, and the virulence of eight or ten different fluids compared in a single animal. The results of five series of experiments made with this technique can be summarized as follows:

When a medium composed chiefly of chicken serum and Tyrode solution and containing no fresh tissues is inoculated with filtered extract of Rous and other sarcomas and incubated for 48 hours, it never produces a tumor after being injected into chickens. The virus has apparently been destroyed or at least has lost activity. But, in a solid medium, composed chiefly of serum and Tyrode solution and containing fragments of fresh tissues, the virus is found to increase readily, as shown in the first series of experiments. In the course of 15 months, the experiments have been repeated many times, with identical results. Flasks containing embryo pulp or leucocytes inoculated with filtered extract of sarcoma are used to keep on hand a constant supply of the Rous virus. The cultures of monocytes inoculated with the filtered extract often assume the appearance of the cultures of Rous sarcoma. They may also remain normal to all appearance despite the circumstance that the virus is multiplying within the medium. It is not certain that the activity of Rous virus is always accompanied by cell lesions, but there is no doubt that its increase depends on the presence of fresh tissues within the medium.

The disappearance of the Rous virus from a medium that does not contain any fresh tissue may be interpreted as follows: the agent has been destroyed; or it is still present in a concentration lower than 1 in 50,000, which is the concentration required to produce a tumor even in the more susceptible chickens; or, according to the hypothesis of Gye,[4] it is present in an inactive form. In the above experiments, the fresh tissues added to the medium might conceivably have enabled the virus to keep its full activity, through supplying the conditions requisite therefor, or they might merely have furnished an activating substance. The value of Gye's hypothesis was tested in a series of experiments. The results indicate that the tumor-producing virus present in the cultures was not composed of two parts, an inactive part

multiplying in the medium, and an activating part supplied by the tissues.

In another series of experiments, the relations between the reproduction of the virus and the quantity of the tissues contained in the medium were studied. The presence of a small fragment of leucocytic film or spleen tissue was sufficient to prevent the virus from disappearing. Approximately 1 c.mm. of spleen tissue in 3,000 c.mm. of medium may on occasion maintain a concentration of Rous virus in this fluid sufficient to produce a tumor upon inoculation into chickens. But this rarely happens. Generally when the medium contained only one fragment of spleen or leucocytic film, or 1 drop of embryonic pulp, the virus disappeared rapidly. When the quantity of tissue was from five to nine times larger, an abundant production of virus was practically always found. It became obvious that the quantity of active virus present in a medium containing multiplying cells depends upon the amount of tissue in the medium.

In the fourth series of experiments, the kind of cells needed for the multiplication of the virus was ascertained. Rous virus was found to disappear rapidly from the fluid of cultures of fibroblasts, while it multiplied readily in cultures of leucocytes, the total volume of both tissues being approximately the same. It should be remembered that strains of fibroblasts obtained from Rous and other sarcomas very rarely produce tumors upon inoculation into chickens,[6] while the inoculation of cultures of macrophages from the same tumors practically always determines their appearance.[7]

The fifth series of experiments showed that the cell metabolism is an important factor in the reproduction of the virus. When the activity of tissues had been suppressed or very much decreased by freezing, no virus was produced, while it multiplied readily in the control. The lack of oxygen for a period of 24 or 48 hours stopped cell proliferation, and at the same time the production of Rous virus ceased. However, the fluid of some of these dead or inactive cultures, after 6 days incubation, was still able to give rise to a small tumor upon inoculation into a chicken. There is an evident relation between the

[7] Carrel, A., *Compt. rend. Soc. biol.*, 1924, xci, 1067.

proliferating activity of the tissues and the production of the virus. But the agent may persist for several days in association with dead tissues.

It may be concluded that the reproduction *in vitro* of the active virus depends on the presence of fresh tissues in the culture and upon the quantity, the activity, and the nature of the cells contained in the medium.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

V. (*a*) Influence of Lymph-Soluble Tissue Materials on the Significance of the Coloration with Some Phthalein Indicators.

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Living tissues of many sorts can readily be stained with indicators of the phthalein series;[1,2] and one can follow local alterations in tissue reaction by means of the color changes. Not infrequently the alterations become pronounced, as in the condition to which we have given the name "outlying acidosis."[3] Even in the healthy animal there would appear to be marked differences in the reaction of some of the organs, judging from the hues they manifest when stained.[2] But do the observed hues mean what they would if encountered under controlled conditions? And are the color alterations which occur in the tissues under abnormal circumstances to be taken at face value, or is this value wholly relative? The present paper and a succeeding one are concerned with these questions.

The phthaleins are less subject to error than are most indicator substances; yet there can be no doubt that in the case of some of them errors of considerable magnitude occur on occasion. True, Clark and Lubs[4] report that salt errors are slight, that phenol red and brom cresol purple in Dunham's solution, beef infusion, and peptone broths behave nearly as they do in buffer solutions, and that brom cresol purple can safely be utilized in the titration of media containing milk; but Clark has been careful to emphasize the fact that indicator may be removed from the field of action by proteins in solution,[5] with result in erroneous colorimetric

[1] Rous, P., *Science*, 1924, lx, 363; *J. Exp. Med.*, 1925, xli, 451.

[2] Rous, P., *J. Exp. Med.*, 1925, xli, 739.

[3] Rous, P., and Drury, D. R., *J. Am. Med. Assn.*, 1925, lxxxv, 33.

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

readings. Kolthoff[6] states that the phthaleins give dependable readings in the presence of the decomposition products of proteins but cannot be used when a solution contains proteins as such. The data responsible for this dictum are not presented. Cullen[7] has successfully employed phenol red for the determination of the pH of blood; but he found dilution necessary to rule out the influence of the serum proteins upon the virage, and his method is not to be relied upon in all pathological conditions.[8] Jaumain[9] has pointed out that brom thymol blue is subject to gross error as an indicator when in contact with serum, even if this latter is in high dilution; though he further noted that with fluids containing some other proteins (gelatin, white of egg) the colorimetric error is relatively inconsiderable

The literature on factors affecting the dependability of the phthaleins seems astonishingly scant, when one considers the popularity of these indicators. But the observations cited suffice to give grounds for caution in the interpretation of the hues that they assume when distributed through the living body. Some theoretical objections to the employment of indicators in the determination of the reaction within living organs will be considered in a succeeding paper.

Our studies have been restricted to three phthaleins possessing ranges that experiment has shown to be well adapted for a color differentiation of tissues on the basis, presumably, of differences in reaction. These three are phenol red, brom cresol purple, and chlor phenol red. Brom thymol blue possesses the proper range for *in vivo* work but is so highly toxic that it cannot be used;[2] and consequently, its errors, as demonstrated by Jaumain, may be dismissed from account in the present connection.

Influence of Phosphate Buffers on the Tissue Coloration.

We made, to begin with, a few observations on the hues of the indicators in solutions of egg albumen or gelatin as compared with potentiometric readings on the same material. But it was evident that one could not generalize from data thus obtained. Only by utilizing ingredients of the tissues themselves for the work could one hope to control in some part the findings in the living creature. Consequently tissues and tissue derivatives were employed in the later experiments.

[6] Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1923.

[7] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

[8] Bennett, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxiii, 114, 115.

[9] Jaumain, D., *Compt. rend. Soc. biol.*, 1925, xciii, 860.

Mice were injected with phenol red or brom cresol purple; they were exsanguinated under ether when staining had become pronounced; and slices of several of the organs were placed in phosphate buffer solutions (Sörensen) of known pH. The color assumed by the slices after they had been immersed for some hours in the buffer fluid was compared with that of the fluid itself into which the stain had diffused,—far more slowly and imperfectly, by the way, than into water or salt solution. Needless to say the tissue slices had to be thin, else interior conditions, not the surrounding medium, largely determined their color; and the buffer mixtures had to be sufficiently concentrated for their reaction to prevail everywhere. In the experiments as at length elaborated N/5 solutions of the phosphates were used and in them were placed 25μ to 100μ sections of liver, as prepared with the freezing microtome, small pieces of shredded tendon, thin sheets of connective tissue, as such, and of cartilage from the ear. In some cases the liver had been washed out with salt solution. The other tissues were always practically bloodless. To render the staining more pronounced a small amount of the phthalein material with which the animals had been injected was often added directly to the buffer solutions.

In brief it was found that cartilage, connective tissue, and tendon, stained with phenol red during life and placed in strong buffer solutions, manifested the same hues to all intents and purposes as did the latter when colored with the dye. They behaved in this respect like so much absorbent paper stained with indicator. Such trifling deviations from the tint of the medium as came to attention were obviously due to the color proper to the tissue itself. No tests were made with chlor phenol red or brom cresol purple, for the reason that when stained with these dyes under the conditions of life, the tissues just mentioned assume hues indicative of a reaction beyond the available phthalein range, on the alkaline side.

The color of sections of liver stained with brom cresol purple or chlor phenol red corresponded less well with those of the surrounding buffer solutions. Preparations submitted to buffers of reactions that ensured the presence of a considerable proportion of the indicator in acid form showed deeply stained sections, the hue being that associated with a frank acidity, whereas in more alkaline solutions, with the indicator all, or nearly all, in the alkaline form, it dissolved out with result that there was either no staining or an anomalous one which might conceivably have been the combined result of a slight accumulation upon the liver slices of such portion of the dye as was present in the acid form and a suffusion of them with buffer contain-

ing the dissolved, alkaline form of the dye. The results suggested that the intense staining of some of the tissues during life might possibly be referable to the accumulation upon them of the undissociated portion of an injected phthalein out of body fluids in which the dissociated and undissociated forms of the indicator were circulating together. Kendall[10] had already noted that slight acidosis increases the absorption of phenol red by the tissues, as also that they hold it more firmly. To widen the observations some further tests were performed.

Mice were totally exsanguinated under ether by the introduction of warmed Ringer's solution into the beating heart after the inferior vena cava had been severed. The kidneys and portions of the liver and skeletal muscle were weighed and ground separately in a mortar with washed sand. The material deriving from each organ was separated into two portions which were mixed, the one with a large excess of N/5 phosphate buffer of pH 5.29, the other with a similar buffer solution at pH 7.17. To these buffers a phthalein solution had been added in identical proportion. The tissue was distributed in the fluid by shaking and the mixture poured out into a Petri dish so large that the tissue and sand formed but a thin sheet over the bottom. After 5 hours at room temperature the material was again collected into a tube, and centrifuged at high speed until the sediment was firmly packed and nothing more would come down. The supernatant fluid was pipetted off as completely as possible; 1 cc. of it was diluted with many times its bulk of water alkalinized by the addition of 1 cc. of 40 per cent NaOH for every 99 cc.; and the entire sediment was similarly treated, on the basis that all of the ground tissue originally employed was present therein and that 1 gm. of this tissue had a bulk of 1 cc. After 24 hours or more in the ice box, to allow for complete extraction of the phthalein, readings of the amount of it in sediment and supernatant fluid respectively were made with the Duboscq colorimeter against a standard containing the appropriate phthalein in known amount. The alkaline solution was too weak to destroy the phthalein; and none had been adsorbed upon the sand. It was regularly noted that the fragments of ground tissue submitted to the buffer at pH 7.17 became somewhat swollen, gelatinous, and sticky, whereas those in the acid buffer remained discrete.

Besides the organs above mentioned, portions of adipose tissue were employed in the work but they lent themselves poorly to the purpose in hand, most of the fat rising to the surface in the centrifuge instead of packing, while furthermore the supernatant fluid was so turbid as to give but a poor match in the colorimeter. It will suffice to say of the results that they accorded with those yielded by the other tests in showing that much more phthalein became fixed on the tissue in the

[10] Kendall, E. C., *J. Am. Med. Assn.*, 1917, lxviii, 343.

acid menstruum. Even under these favorable circumstances, though, relatively little indicator accumulated upon the fat itself.

In the tests made with brom cresol purple so much phthalein had been provided as compared with tissue that the amount taken out of solution by this latter was inconsiderable as compared with that left

TABLE I.

The Distribution of Phthaleins in Mixtures of Tissue with N/5 Phosphate Buffer Solutions.

Tissue.	Buffer reaction.	Amount of indicator in the undissolved tissue materials.							Remarks.
		Brom cresol purple.			Cresol red.		Thymol blue.		
		Cc. of buffer solution per gm. of tissue.	Concentration.	Proportion of total for mixture.	Concentration.	Proportion of total for mixture.	Concentration.	Proportion of total for mixture.	
	pH			*per cent*		*per cent*		*per cent*	
Liver.	7.17	13.6	1	—	1	30.6	1	33	1 cc. of 1 per cent cresol red and thymol blue at approximately pH 7.4 was added to 44 cc. and 50 cc. respectively of buffer solution. 1 cc. of 2 per cent brom cresol purple was added to 30 cc. of the buffer solution that was to be mixed with liver tissue, and 1 cc. to 50 cc. of that employed with the other tissues. 20 cc. of the solution colored with cresol red or thymol blue was used for every gm. of tissue.
	5.29	13.6	4.04	—	2.6	66	2.35	76	
Kidney.	7.17	37.3	1	—	1	46	1	41	
	5.29	37.3	5.16	—	2.24	81	2.24	72	
Muscle.	7.17	18.75	1	—	1	29	1	49	
	5.29	18.75	2.95	—	2.09	65	1.76	72	

behind; but in the later experiments with cresol red and thymol blue the quantity added to the buffer solutions was better judged, and it was possible to check the influence of weak acidity and alkalinity respectively, not alone by the proportion of dye actually found in the sediment, but by the amount of it remaining in the fluid. The discrepancies between the findings with sediment and fluid respectively

(Table I) can be sufficiently explained by difficulties in the colorimetric readings, though it is conceivable that there was some destruction of phthalein by the tissues similar to that Kendall has noted in the case of phenol red.[10] Experiments to be described further on speak against the importance of any such occurrence in the present instance, as do further the conditions of the tests themselves. The destruction, of phenol red at least, is largely conditioned upon an oxygen lack, and our preparations were well oxygenated.

On consulting the table (Table I) it will be seen that, in consonance with our previous observations upon sections of liver, far more brom cresol purple accumulated upon the tissues out of the acid buffer solution than out of the alkaline one, several times as much indeed. Clark's chart[5] shows that a watery solution of brom cresol purple at pH 5.29 contains more than 75 per cent of the indicator in the undissociated, or acid, form; whereas at pH 7.17 less than 20 per cent is in this condition. To determine whether these proportions had been responsible for the outcome of our tests cresol red was utilized, a phthalein which is all in the acid form at pH 5.29 and nearly all (about 90 per cent) at pH 7.17. The proportion of cresol red taken out of the buffer solution at pH 5.29 by the tissues averaged more than twice that at pH 7.17, as shown by the titration of both fluid and sediment. This finding suggested that the results with brom cresol purple were referable, not to the proportion of phthalein present in acid form in the buffer-tissue mixtures but to some effect of the phosphate solutions upon the tissues themselves, a possibility further supported by the differing state of the tissues in the two buffers, as above described. That such was the actual case was shown by tests with thymol blue, an indicator which is practically all in the alkaline form both at pH 5.29 and pH 7.17. The results with it were essentially the same as of those with cresol red, and, due allowance being made for the differing quantities of indicator provided, as of those with brom cresol purple. Approximately twice as much indicator accumulated on the tissues at pH 5.29 as at pH 7.17.

Influence of Lymph-Soluble Tissue Materials on the Phthalein Colors.

This demonstration of the extraneous character of the findings in stained tissues exposed to artificial buffer solutions led us to abandon

their use and to employ fluids that might yield a closer approach to conditions as they exist within the body. Two sets of experiments were devised, one to control the influence upon the phthalein colors of substances brought into solution out of the tissues, the other,—dealt with in a succeeding paper,—to demonstrate the effect of the remaining, undissolved, tissue materials upon the color of indicators adsorbed or in other ways accumulated upon them.

In tests of the sort first mentioned a concentrated extract of fresh tissue was made, phthalein added, and readings carried out with the colorimeter and the potentiometer upon the same specimens. In some tentative observations water or salt solution served for the extraction; but lymph of the animal furnishing the tissue was ultimately adopted as a more natural menstruum. In order to bring the reaction of the extracts within the ranges of brom cresol purple and chlor phenol red it was necessary to acidify them slightly. This was done by adding dilute lactic acid. A summary of the colorimetric and potentiometric comparisons obtained by the method is given in Table II.

The protocols which follow, and which find further record in the table, are those of all of our experiments in which sound working conditions were attained. These involved among other things a practically complete absence of hemoglobin from the extracts, the development in the latter of acidity without precipitation when lactic acid was added, a strong and constant light source for colorimetric purposes, and electrodes which did not show a deterioration on contact with the extracts, such as Cullen has noted in his studies with serum.[7]

To prepare the extracts the kidneys and one or more lobes of the liver were perfused with water, or 0.9 per cent salt solution, or lymph,—according to whichever was to be used for the extraction,—followed in some instances, by an injection of air to empty the vessels. The perfusions were made into the renal arteries and branches of the portal vein of the organs as removed from the etherized animal. The most satisfactory washings were those carried out with lymph. When it was used all the blood was readily flushed out and the tissue appeared unaltered otherwise, whereas when water or salt solution had been employed there was frequently some hemoglobin left behind and the tissue itself sometimes appeared swollen. In an instance in which salt solution had been employed for the washing but lymph for the extraction, a prompt precipitation occurred in extracts to which lactic acid was added. This did not take place when lymph had been used at every step in the preparation.

In order to get sufficient lymph for our purposes it was necessary to use large dogs. To ensure that it should be clear the animals were not fed for 24 hours before the operation; but they were given 500 to 750 cc. of water by gavage a few

TABLE II.

Comparative Colorimetric and Electrometric Determinations of the Reaction of Tissue Extracts.

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
Liver + Water + Phenol Red.											
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Experiment V.	*a*	633	6.37	Between	642	6.52	Approximately	Fairly easy.			
Guinea pig livers. Tubes *a* and *b* and their controls were derived from different animals.		645	6.56	6.37 and 6.56.			−0.06.				Saturated calomel electrode; N/10 HCl = 307 millivolts.
	b	645	6.56	6.56	634	6.39	+0.17				
Experiment VI.	*a*	633	6.37	6.37	632	6.36	+0.01	Easy.			
Dog liver.	*b*	633	6.37	Just below 6.37.	631	6.34	None?	"			Saturated calomel electrode; N/10 HCl = 307 millivolts.
	c	633	6.37	Just above 6.37.	631	6.34	Slightly more than +0.03.	"			
Liver + Salt + Phenol Red.											
Experiment VI. Dog liver.		645	6.56	6.56	629	6.31	+0.25	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.

Liver + Lymph + Phenol Red.

Experiment IX. Dog liver. Double strength extract.	*a*	657 666	6.70 6.84	Between 6.70 and 6 84.	658	6.71	Approximately +0.6.	Fairly easy.	1.62	10.1	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	666	6.84	6.84	659	6.73	+0.11	Fairly easy.	1.70	10.6	
		646	6.52	6.52	653	6.63	−0.11	Difficult.			
Experiment X. Dog liver. Drifting necessitated frequent changing of electrodes. Double strength extract. Twice as much P.R. in extracts and buffers as usual.	*a*	634.5 646	6.33 6.53	Between 6.33 and 6.53.	652.5	6.62	Approximately −0.19.	Very difficult.	1.605	10.05	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	622	6.13	6.13* or less!	652.5	6.62	−0.49 or more.	Very difficult.			
	a	647	6.54	6.54	640	6.42	+0.12	Very difficult.	1.68	10.5	
	b	647 657	6.54 6.70	Between 6.54 and 6.70.	638	6.39	Approximately +0.22.	Very difficult.			
Experiment XI. Dog liver. Double strength extract.	*a*	655 664	6.66 6.81	Between 6.66 and 6.81.	645	6.50	Approximately +0.24.	Fairly easy.	1.29	8.05	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	646 655	6.51 6.66	Between 6.51 and 6.66.	644.5	6.49	Approximately +0.09.				

The "half strength" extracts were made with twice as many cc. of lymph as there were gm. of tissue, the "double strength" with half as many cc. as gm. Ordinarily a cc. to gm. ratio was employed.

* The specimen contained so much phenol red that it was possible to make colorimetric readings which involved this degree of acidity.

TABLE II—*Continued.*

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
Liver + Lymph + Brom Cresol Purple.											
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Experiment XII.	*a*	583	5.48	Between	589	5.59	Approximately	Easy.			
Dog liver. 5 *gtt.* lactic acid.		602.5	5.81	**5.48** and **5.81.**			+0.06.				
									1.46	9.15	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	583	5.48	Slightly less than **5.48.**	577	5.39	Less than +0.09.	Easy.			
Experiment XV.	*a*	595	5.69	Just above **5.69.**	611.5	5.96	Less than −0.27.	Easy.			
Dog liver. 5 *gtt.* lactic acid. Drifting necessitated frequent changing of electrodes.									1.11	6.96	
	b	603.5	5.82	**5.82**	605	5.86	−0.04	Easy.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
Double strength extract; 5 *gtt.* lactic acid.	*a*	623	6.14	**6.14**	—	—	—				
	b	623	6.14	**6.14**	619.5	6.09	+0.05	Easy.	1.35	8.45	
	a	623	6.14	Just above **6.14.**	620	6.10	Slightly more than +0.04.	Easy.			
Liver + Lymph + Chlor Phenol Red.											
Experiment XII. Dog liver. Double strength extract; 2 *gtt.* lactic acid.	*a*	619	6.08	**6.08**	621	6.11	−0.03	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.

Experiment XII—*Continued.* The same; but 3 *gtt.* lactic acid.	*a*	582.5 602	5.49 5.81	Between **5.49** and **5.81**	613	**5.98**	Approximately −0.33.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	582.5 602	5.49 5.81	Between **5.49** and **5.81.**	609	**5.91**	Approximately −0.26.	Rather difficult.			
Experiment XV. Dog liver. 5 *gtt.* lactic acid.		622	6.12	Just below **6.12.**	604	**5.84**	Less than +0.28.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
Kidney + Water + Phenol Red.											
Experiment V. Guinea pig kidney. Half strength extract.		633	6.37	Just above **6.37.**	634	**6.39**	−0.02	Fairly easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Experiment VI. Dog kidney. Half strength extract.		645 655	6.56 6.73	Between **6.56** and **6.73.**	650	**6.61**	Approximately +0.04.	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Kidney + Salt + Phenol Red.											
Experiment VI. Dog kidney. Half strength extract.		645	6.56	**6.56**	634	**6.39**	+0.17	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Kidney + Lymph + Phenol Red.											
Experiment IX. Dog kidney.		677	7.02	Just below **7.02.**	673	**6.95**	None?	Fairly easy.	1.055	6.60	3.5 N calomel electrode; N/10 HCl = 311 millivolts.

TABLE II—*Concluded.*

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
Kidney + Lymph + Phenol Red—*Continued.*											
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Experiment XI.	*a*	655	6.66	Between	653	6.61	Approxi-	Fairly			
Dog kidney. Extra strength extract. Two-thirds as		664	6.81	6.66 and 6.81.			mately +0.13.	easy.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
many cc. of lymph as there were gm. of tissue.									1.28	8.00	
	b	655	6.66	Between	653	6.61	Approxi-	Fairly			
		664	6.81	6.66 and 6.81.			mately +0.13.	easy.			
Kidney + Lymph + Brom Cresol Purple.											
Experiment XII.	*a*	583	5.49	Between	584	5.51	Approxi-	Easy.			
Dog kidney. Double strength extract; 5 *gtt.* lactic acid.		602.5	5.81	5.49 and 5.81.			mately +0.14.				3.5 N calomel electrode; N/10 HCl = 311 millivolts.
									1.12	7.0	
	b	583	5.49	Between	585	5.52	Approxi-	Easy.			
		602.5	5.81	5.49 and 5.81.			mately +0.13.				

Experiment XV. Dog kidney. 4 *gtt.* lactic acid.	*a*	595 603.5	5.69 5.82	Between **5.69** and **5.82.**	611	**5.95**	Approximately −0.20.	Easy.			
									0.74	4.6	
	b	587	5.56	Slightly above **5.56.**	607	**5.89**	Less than −0.33.	Easy.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
Double strength extract; 5 *gtt.* lactic acid.	*a*	595 603.5	5.69 5.82	Between **5.69** and **5.82.**	—	—					
	b	587 595	5.56 5.69	Between **5.56** and **5.69.**	597	**5.72**	Approximately −0.10.	Easy.	0.90	5.6	
	a	603.5	5.82	**5.82**	593.5	**5.66**	+0.16	Easy.			
Kidney + Lymph + Chlor Phenol Red.											
Experiment XV. Dog kidney. 4 *gtt.* lactic acid.		622	6.12	Just below **6.12.**	604	**5.84**	Less than +0.28.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.

minutes prior to it. Under ether the thoracic duct was cannulated and lymph collected during several hours until, in some cases, 200 cc. or more had been got. This was rid of the thin clot by stirrings with a rod, and centrifuged when it contained red cells. The renal arteries were now tied, then the renal veins, and both kidneys were rapidly removed and perfused through the arteries under slight pressure. A 20 cc. syringe was used, both to inject fluid and to force air in after it. Leakage backward from the needle was prevented by clamping it and the enveloping vessel wall between the rubber-covered jaws of a hemostat forceps. During the washing the kidneys were gently kneaded. Next the animal was bled to death by cutting the inferior vena cava above the diaphragm; and the lateral mass of the liver, comprising the right lateral and caudate lobes, was perfused with lymph through the branch from the portal vein.

To prepare the extracts the pallid tissues were cut into blocks which were squeezed between folds of washed and dried gauze to remove free fluid and then forced through a barrel-and-plunger sieve provided with round openings less than 1 mm. in diameter. The cortex only of the kidney could be used, the medulla proving too tough for easy pulping. The pulp was mixed with lymph, or salt solution, or water, as the case happened to be, in the proportion, ordinarily, of as many cc., or half as many, as there were gm. of tissue. The mixtures were shaken violently in a machine for 20 minutes and centrifuged at high speed for from ¾ of an hour to an hour, that is to say until everything had come out of suspension that would. The supernatant fluids,—the extracts,—were pipetted off and distributed in portions of 2½ or 3 cc. in colorimeter tubes. To some of the portions 0.2 cc. or more of a dilute solution of phthalein indicator was added, while others were kept for control purposes. Ordinarily the phthalein had been made up in 1 per cent or 2 per cent solution isotonic with 0.9 per cent NaCl and at pH 7.4; and it was further diluted with water to 0.1 per cent or 0.2 per cent. A number of duplicate mixtures of tissue extract and phthalein were usually made. It was deemed best in most cases to add the indicator, as also any lactic acid required, to the small portions of extract as such rather than to the whole quantity prior to distribution, since in this way opportunity was given for some slight individual variation in the pH of the specimens. N/15 phosphate buffers (Sörensen) were employed in the colorimetric readings, and phthalein was added to them in the same proportion as to the extracts. The readings were made in the usual way by contrasting the color of a tube of the tinted extract plus one of water, in an ordinary colorimeter block, with that of a tube of untinted extract plus one of tinted buffer. Tubes of 12 mm. diameter, carefully cleansed and dried beforehand, were employed. The readings were at 38°C., like those in the potentiometer. Always the control tubes of buffer and of extract were warmed at the same time as the tinted extract; and when lactic acid had been added to the latter the same amount was added to the control specimen as well. The acid caused some gradual change toward opacity in the already turbid extract and for this reason those tinted duplicate specimens containing it which had been separately warmed for reading, were usually not read against the same control specimen, warmed for both, but against different ones treated like themselves.

The phthalein hues could be much more readily distinguished in the kidney extracts, which were a creamy buff, than in the brownish buff liver extracts. All were far too opaque for color comparisons by daylight. Instead an arc light was used having a lens of 9.5 cm. aperture that rendered the rays parallel.

In the earlier work a single observer carried out both sets of readings, the colorimetric series first, and then, as rapidly as might be, the electrometric ones, on the same series of specimens. Since the potentiometer readings were strictly quantitative in character it was felt that the personal equation as involved by a knowledge of the colorimetric pH could have no part in them. Only a short time elapsed between the two series of readings as compared with the period during which the extracts had been in preparation; and such extracts were found to alter little in reaction on standing for the necessary period at room temperature. Nevertheless, as a check on the influence of this factor, the order in which the extracts were tested electrometrically was varied from that of the colorimeter observations, some specimens being tested at once after the color reading and other duplicate ones not until a considerable time had elapsed. The readings on the liver specimens of Experiment VI, Table II, furnish an instance in point. During the later work (Experiments IX to XV inclusive), a potentiometric reading on each specimen was made immediately after the colorimetric and by a different observer unapprised of the color findings. Since the duplicate specimens were regularly tested in succession by both methods and they were found to differ but little in pH, it can safely be assumed that the time element as involved in a rapid transfer from the colorimeter to the potentiometer had no hand in the results. As a further check on the influence of the time factor, however, two colorimetric readings on the same specimen were sometimes made, with an interval between, and an immediate potentiometer test after the second one. The reactions of the tinted buffer solutions used in the comparisons were determined electrometrically.

Cullen[7] has described the deterioration of electrodes which takes place when they are brought in contact with serum. The difficulty sometimes made itself manifest with our tissue extracts, yet much less often than had been apprehended. A number of electrodes were kept ready and, though occasionally several in succession showed "drifting," one was usually found with which the few observations of the individual experiment could be run off. All readings were at 38°C. All were carried out on the afternoon that the extracts were prepared, except, in some instances, those on the tinted buffers. In these instances the buffer tubes were kept overnight in the ice box prior to the potentiometric determinations.

It is conceivable that there was some small escape of CO_2 from the portions of extract after they had been read colorimetrically, when they were shaken in the hydrogen atmosphere of the potentiometer chamber. But this can scarcely have been an important source of error, since the CO_2 content of the extracts must have been slight, owing to their acid reaction and the frequent aeration involved in the preparation and handling of them.

I. Tests with Phenol Red.—Liver and kidney tissues of the living animal are colored yellow by phenol red, the reaction lying to all appearance outside of the

range of the indicator on the acid side, even when the latter is present in high concentration, under which circumstances its range may extend as far down as pH 6.10. But as Michaelis and Kramsztyk[11] and others have noted, liver and kidney extracts in water or salt solution have a relatively high pH, as determined electrometrically, usually one between pH 6.40 and pH 6.80. In agreement with this finding our extracts all had a reaction well within the range of phenol red in the concentrations used, and beyond the ranges of brom cresol purple and chlor phenol red on the alkaline side. The extracts made with the lymph, which itself had a reaction close to pH 8.0, were not more alkaline than those with water or salt solution (Table II), a fact which shows that dissolved tissue derivatives were present in sufficient abundance to determine the reaction.

The pronounced buff of the liver extracts rendered color determinations with phenol red relatively difficult, and, in the case of the double strength extracts, far from precise (Table II).

Experiment V.—The liver and kidneys of one guinea pig and the liver of another were washed and extracted with twice-distilled water. The extracts from the two animals were kept separate. Those of the hepatic tissue were made on the basis of 1 cc. of fluid for every gm. of tissue, while twice as much fluid was added in the case of the kidneys. To each 3 cc. portion, 0.025 cc. of 0.2 per cent phenol red was added excepting in the case of Tube *b*, liver, which received, like the appropriate buffers, 0.05 cc.

Experiment VI.—One kidney of a 7 kilo dog was washed out and extracted with salt solution, the other with distilled water; and so also with two lobes of the liver. Extraction was done with the appropriate fluids, using the same proportions as in Experiment V. To the 2.5 cc. specimens 0.02 cc. of 0.2 per cent indicator was added.

In these experiments the extracts were less concentrated and turbid than in the later ones. Colorimetric readings proved relatively difficult in the latter.

Experiment IX.—From the thoracic duct of a dog of 18½ kilos, 130 cc. of lymph was collected. One of the kidneys and the lateral mass of the liver were washed with it, and extracted, in the proportion of 1 cc. lymph to 1 gm. in the case of the renal tissue and most of the hepatic. To part of the latter only half as many cc. lymph were added as there were gm. of tissue. This extract is designated in the table as double strength. Unfortunately not enough phenol red was used to permit of precise readings. The phthalein was mixed with all of the extracts in bulk, 0.1 cc. of 0.1 per cent solution for every 2.5 cc.

Experiment X.—Only 110 cc. of lymph was obtained from this 14½ kilo dog, and there was some blood left in the one kidney that was washed, despite the injection of air after the perfusion. Extraction was made from the renal tissue on the basis of two-thirds as many cc. of lymph as there were gm. of tissue. Two liver extracts were made, one in the cc.-gm. ratio, the other with ½ cc. to a gm.; and to 2.5 cc. portions of these, respectively, 0.2 cc. and 0.1 cc. of a 0.1 per cent

[11] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

solution of phenol red were added. The kidney extract received 0.1 cc. The color readings proved exceptionally difficult, and "drifting" necessitated several changes of the electrodes.

Experiment XI.—Since only 60 cc. lymph was obtained from the dog of 16½ kilos it was found necessary to wash out the kidneys and liver with 0.9 per cent salt solution. This was expelled from the vessels as completely as possible with air. The liver material was extracted with half as many cc. of lymph as there were gm. of tissue; and two-thirds as many cc. were employed as gm. of renal tissue. To every 2.5 cc. portion of extract 0.2 cc. of 0.1 per cent phenol red was added.

II. Tests with Brom Cresol Purple and Chlor Phenol Red.—Portions of the same extracts were tinted with these two indicators, so the preparations for both sets of tests can be described together. The virages of the liver parenchyma and of the kidney cortex stained during life are ordinarily well down toward the acid side of the ranges of both brom cresol purple and chlor phenol red.[2] But, as might have been predicted from the readings with phenol red, the extracts had reactions so far toward the alkaline side that the addition of acid was necessary to bring them within the range of the phthaleins. N/4 lactic acid was employed, drop by drop with shaking after each, until the changed hue indicated that readings had become possible. The tubes containing acidified extract tinted with brom cresol purple showed dichromatism, appearing green by ordinary light; but in the colorimeter block there was no suggestion of this color and comparisons with the buffer standards were readily to be made. They proved more difficult with chlor phenol red owing to the fact that the orange hues exhibited by this indicator on the acid side of its range predominated less plainly over the buff natural to the extracts.

Experiment XII.—Lymph to the amount of 120 cc. was collected from a dog of 16 kilos. A kidney and the lobe mass of the liver were successfully washed with some of it, and the perfusion fluid forced out of the vessels with air. Two liver extracts were made, on the gm.-cc. and gm.-½ cc. basis respectively. The kidney extract had the latter basis. To 2.5 cc. portions of each, 0.2 cc. of a 0.1 per cent solution of either chlor phenol red or brom cresol purple was added, followed by lactic acid.

Experiment XV.—From a dog of 10 kilos, 230 cc. of lymph was obtained. Both kidneys and the lobe mass of the liver were perfused with the fluid and further emptied by injections of air. Two extracts were made of each tissue, one with as many cc. of lymph as there were gm. of tissue, the other with half as many. To 2.5 cc. portions of all 0.15 cc. of a 0.1 per cent solution of either brom cresol purple or chlor phenol red was added, followed by enough N/4 lactic acid to bring the color well within the indicator range. A change of electrodes during the potentiometer readings was necessitated by "drifting," and some specimens were lost in consequence.

When the table is consulted (Table II) it will be seen that the colorimetric and potentiometric determinations accorded less closely in

some experiments than in others, owing, as was evident at the time, to difficulties in reading the color of very turbid solutions naturally buff in hue and containing phthaleins which were themselves orange or orange-red at the reaction studied. All in all though, the readings corresponded far more closely than might *a priori* have been expected. Phenol red gave pH findings that were for the most part slightly higher than those obtained with the potentiometer, though it is questionable whether this was not due to chance. The differences manifested by individual specimens stained with brom cresol purple and chlor phenol red lay now in this direction, now in that, the deviations approximately balancing one another.

The extracts coagulated when they were boiled and they contained a considerable amount of the tissue nitrogen, as the Kjeldahl figures attest (Table II). According to Grund[12] there is about 2.9 per cent of nitrogen in the liver of the dog and 2.1 per cent in the kidney, nearly nine-tenths of these amounts being present as protein. Some of our extracts contained more than 1.6 per cent of nitrogen. Of this as much as 0.7 per cent may have been derived from the lymph.[13]

SUMMARY.

The present paper is the first of two dealing with experiments which were planned to disclose the meaning of the colors assumed by mammalian tissues when vitally stained with some phthalein indicators. Derivatives of the tissues themselves were employed after tests had shown, in agreement with certain facts in the literature, that inferences from model experiments, so called, would be misleading.

Organs were perfused with water, or salt, or lymph of the same creature and extracted with small quantities of the fluid. The turbid extracts were colored with phthalein, the reaction determined colorimetrically with the aid of an arc light, and the findings were compared with potentiometer readings upon the material. The results of the two methods were in close agreement.

The significance of the data will be discussed in our second paper.

[12] Grund, G., *Z. Biol.*, 1910, liv, 173.

[13] Petersen, W. F., and Hughes, T. P., *J. Biol. Chem.*, 1925, lxvi, 229.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

V. (*b*) Influence of Lymph-Insoluble Tissue Materials on the Significance of the Coloration with Some Phthalein Indicators.

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In a microscopic study of living tissues stained with the phthaleins one sees no sign that the coloration is due to accumulation of the indicator upon special cytoplasmic substances. It is true that under certain circumstances a segregation of phthalein within cell vacuoles may occur;[1] but ordinarily the staining appears diffuse, and only in the case of bone cells and cartilage cells do the findings suggest that there may be some deposition of dye upon the cell surfaces. But needless to say, the evidence of the eye is not conclusive in this matter; nor can it even be deemed of much value when one considers the fact that phenol red is removed on occasion from the field of action as an indicator by proteins in solution. The experiments with tissue extracts described in an accompanying paper[2] may perhaps be taken as constituting a partial check upon the influence of this removal factor upon the colors exhibited by tissues. They tell nothing, however, of the possibility of errors due to an accumulation of phthalein upon tissue materials insoluble in lymph, salt solution, or water. True, such materials were doubtless present in suspension in our turbid preparations, but only in small quantity. To learn something of the partition of the phthaleins between extract and tissue residue an experiment was performed as follows:

[1] Rous, P., *J. Exp. Med.*, 1925, xli, 379.

[2] Drury, D. R., and Rous, P., *J. Exp. Med.*, 1926, xliii, 669.

Distribution of the Phthaleins to Extract and Residue.

Tissues of several sorts, practically blood-free, were ground with sand and mixed with lymph of the same animal in the proportion of 1 cc. for every gm. The lymph had been colored with phthalein. The mixtures were shaken; centrifuged until the sediment was firmly packed; and the relative proportion of the indicator in fluid and sediment was determined with the colorimeter. Duplicate mixtures were employed, and one series rendered more acid than the other by saturation with carbon dioxide.

About 170 cc. of lymph was collected under ether from the thoracic duct of a mongrel terrier weighing 10 kilos. The kidneys were perfused with it, as well as a liver lobe, and the kidney vessels were emptied by blowing air through them. After the animal had been bled to death from the inferior vena cava portions of the pancreas, voluntary muscle, fat, and connective tissue were also taken. They were nearly bloodless. The connective tissue was derived from the subcutaneous regions of the thigh, the muscle was from the upper leg, and the fat was partly retroperitoneal, partly omental. The materials were cut up, pressed between layers of gauze to remove the free fluid, and 3 gm. portions were ground with sand and mixed with 3 cc. of lymph, except in the case of the connective tissue 2.5 gm. of which was used with an equal quantity of fluid. To some of the portions 0.2 cc. of a freshly made 1.0 per cent solution of brom cresol purple was added, to others a like amount of a 1.0 per cent solution of chlor phenol red. Both indicator solutions were at pH 7.4, and had been rendered isotonic with 0.9 per cent NaCl by the addition of this salt. The sand and lymph tissue mixtures were placed in long test-tubes, which they but one-third filled, shaken for 20 minutes at room temperature, and centrifuged without change of the container for ½ hour at high speed. Prior to the shaking one series of specimens with each indicator had been saturated with CO_2 by repeatedly running the washed gas into the tubes and briefly shaking them by hand. When the failure of the treated mixtures to change color further, despite the fact that the limits of the ranges of the indicators had not been reached, attested to saturation with the gas, the tubes were stoppered with paraffin corks; and they were not opened until after centrifuging. Meantime the coloration had not altered. The materials stained with brom cresol purple altered under the influence of the CO_2 from purple to a dirty purple-green, and those with chlor phenol red from rose-purple to yellowish pink.

Now the supernatant fluids were pipetted off, carefully measured, and, to 1 cc. of each, 20 cc. of water containing 1 cc. of 40 per cent NaOH in every hundred cc. was added. The entire sediment was treated in the same way. In so doing allowance was made for that portion of the fluid which remained mixed with the original 3 gm. of tissue; and for purposes of dilution it was assumed that the latter had a

bulk of 1 cc. for every gm. Thus if only 2.8 cc. of fluid had been recovered instead of the original 3.2 cc. it was held that 0.4 cc. was mixed with the 3 cc. of tissue capable of holding phthalein, and dilution was made as for 3.4 cc. In the case of some of the tissues, notably the pancreas, more fluid was recovered than had been added to the tissue. In such cases it was assumed for purposes of dilution that the number of cc. of tissue in the sediment was correspondingly less. Since a little of the tissue had been lost in the transfer from mortar to tube, while moreover it had a smaller bulk gm. for gm. than the lymph, owing to its higher specific gravity, the sediment containing it must have been somewhat overdiluted. The material mixed with the alkalinized water was placed in Erlenmeyer flasks, left in the ice box overnight, and then centrifuged, and the color of the fluid from the sediment specimens was read against that from the supernatant specimens and one of these in turn against a standard made from a portion of the original lymph-phthalein mixture which had been kept as such in the cold until the moment for use. The general results of the comparison with the freshly prepared standard sufficed to show that none of the indicator had been destroyed during its contact with tissue or the alkalinized water. Owing to a differing turbidity of the colored fluids derived from sediment and supernatant fluid respectively the determinations could be only approximate in some cases. For a satisfactory reading against the standard an ultimate dilution to 1 in 100 was found advisable. A control test showed that sand of the sort used in the grinding, and present in the sediment, did not accumulate phthalein.

The results find record in Table I. It will be seen that there was a relative accumulation of brom cresol purple upon the sediment of liver, kidney, pancreas, and muscle, but not on the adipose and connective tissues. If allowance is made, in the case of the tissues first mentioned, for the amount of lymph remaining with the sediment, and for the fact that the actual bulk of the sedimented tissue was less than has been assumed, the accumulation upon the undissolved tissue materials will prove somewhat greater than our figures show. At most though it cannot have been considerable. Brom cresol purple dissolves far less well in fat than in water and to this cause may be attributed the relatively small amount of the phthalein in the adipose tissue, as also that in the connective tissue the specimens of which happened to contain some fat.

Chlor phenol red accumulated upon the liver sediment, but not upon pancreas sediment. The accumulation of brom cresol purple upon the sediment of liver, kidney, and muscle appeared to be somewhat greater when the reaction of the mixtures had been rendered

weakly acid by saturation with CO_2. The difference has interest not only as agreeing in little with the more marked difference noted when the tissues lay in phosphate buffers (Table I of preceding paper), but because, in consonance with Kendall's[3] observations, Grollman[4] has shown that the adsorption of phenol red by proteins is largely

TABLE I.

The Distribution of Phthaleins in Lymph and Tissue Mixtures.

Tissue.	Treatment.	Percentage concentration in terms of the mean.					
		Brom cresol purple.			Chlor phenol red.		
		Supernatant.	Sediment.	Sediment, after correction for admixed lymph.	Supernatant.	Sediment.	Sediment, after correction for admixed lymph.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Liver.		94.8	105.2	107.6	92.5	107.5	107.5
	CO_2	92.5	107.5	110.8	85.1	114.9	115.8
Kidneys.		97.4	102.6	103			
	CO_2	86	114	115.9			
Pancreas.		97.7	102.3	102.3	102.9	97.1	97.1
	CO_2	93.3	106.7	106.7	102.5	97.5	97.3
Muscle.		94.1	105.9	108			
	CO_2	91	109	112.5			
Adipose tissue.		111.1	89.9	83.3			
	CO_2	114.3	85.7	78.1			
Connective tissue.		98.2	101.8	102.8			
	CO_2	113.2	86.8	77.4			

governed by the pH of the medium, being greatest when this medium is slightly acid.

The lymph-tissue-phthalein mixtures of our experiment contained the indicator in about one-third the proportion required on intravenous

[3] Kendall, E. C., *J. Am. Med. Assn.*, 1917, lxviii, 343.

[4] Grollman, A., *J. Biol. Chem.*, 1925, lxiv, 141.

injection to produce a pronounced general coloration of an equivalent weight of living rat.

The observations leave no doubt that under test-tube conditions there is some accumulation of phthalein from the lymph upon those materials of tissue which are not lymph-soluble. Yet inferences concerning the partition of the dyes in the living animal would not be warranted by such findings. Through vital processes cells of some sorts, notably those of the liver and kidney, effect a local accumulation of the phthaleins far more considerable than ever occurs upon dead tissues submitted to lymph containing the indicators; and the intercellular substance of cartilage and tendon become far more deeply tinted than the lymph in which they are bathed.

Influence of Lymph-Soluble Materials upon the Phthalein Colors.

The fact that phthalein accumulates upon undissolved tissue materials *in vitro* renders possible a test of the effect of the latter upon the phthalein color. One need only submit slices of the tissue to a lymph in which the indicator is present and compare their color after a time with that of the fluid in which they are contained. Needless to say, though, the slices must be very thin else the reaction of the lymph cannot be expected to prevail throughout them; and while enough phthalein must be present for the tissues to be well stained by it, not so much can be employed as would hinder colorimetric readings on the fluid.

Portions of the lymph and tissues which furnished the material for the experiment just described were utilized for our initial tests. Several 100μ sections from certain of the organs, cut with the freezing microtome, were placed in a large excess of lymph (2 cc.) to which had been added 0.125 cc. of 0.1 per cent brom cresol purple, enough of the indicator that is to say to give an outspoken purple hue to the fluid, but not an intense one. Two series of preparations were made, with liver and kidney washed by perfusion, and with exsanguinated muscle, pancreas, adipose tissue, and fascia respectively. The fascia specimens were not sections but small rectangles cut with scissors from a sheet of the fascia lata. They were especially suited to color observations, being white, bloodless, close textured, and of an even thinness.

The contents of one series of tubes containing lymph and tissue were saturated with CO_2 according to the procedure described in the experiment on phthalein partition, and the tubes were tightly stoppered with paraffined corks, warmed to

37°C. in the water bath, and maintained at this temperature for 2 hours, kept overnight in the ice box, and again placed at 37°C., this time for 4½ hours. The stoppers had been fixed in place with adhesive.

Immediately prior to the color comparison a layer of clean paraffin oil 1½ cm. thick was run above the lymph, and through this layer the sections were lifted out by means of a fine glass hook for inspection between a mica slide and cover. That the protection afforded by the oil prevented the escape of CO_2 from the material saturated with it was sufficiently shown by the lack of any change in the color of the sections during the manipulations or shortly after them. The hue of each section as flattened under the cover was compared at room temperature with that of the fluid in which it had been immersed. All showed staining, though to differing degrees, that of the adipose tissue being faint; and without exception all manifested the same general hue as the lymph, a hue superimposed though, in the case of the liver and kidney, upon the buffy tint natural to the tissue. To test, roughly, whether more phthalein was present in the material which had lain in the lymph saturated with CO_2 than in that exposed to lymph as such, sections of each tissue from both sources were placed side by side upon a single slide, blotted dry, and watery N/1 sodium carbonate solution was run upon them. The purple color they developed had the same degree of intensity.

That part of the lymph which was employed without other modification than the addition to it of brom cresol purple had a reaction close to pH 8.0 as determined with phenol red and phosphate buffers. It was in other words so alkaline as to be well beyond the range of brom cresol purple. Such being the case no difference between the color of the tissue stained with this indicator and that of the natural buffer medium about it was to have been expected. Both would have appeared purple unless the influence of the tissue materials to cause a phthalein error had been of such magnitude and lain in such direction that at an actual pH of 8.0 the section exhibited a hue indicative of an acidity of pH 6.2 or less. This, of course, did not prove to be the case. Under the conditions brought about by the saturation with CO_2, on the other hand, the test was a delicate one. The reaction of the lymph was changed through the saturation to between pH 5.52 and 5.65, as determined by a comparison with tinted buffers that had been checked with the potentiometer, and the color of the tissue to that manifested by certain of the organs (liver, kidney, pancreas, muscle) when they are vitally stained.

In a second experiment lymph was collected from a 12 kilo dog, portions of the liver and kidney were perfused with some of it as usual, and pieces of the pancreas, voluntary muscle, xyphoid cartilage, tendon from the knee, and fascia lata were taken immediately after the animal had been exsanguinated under ether. Pieces of all these tissues except the fascia lata were largely rid of free fluid by pressure between the folds of a cloth, and they were cut into 100μ sections. The fascia was divided into small rectangles, as in the previous experiment. Since fascia, tendon, and cartilage when stained during life are orange-pink with phenol red, and purple and rose-purple respectively with brom cresol purple and chlor phenol red, it was

important to learn merely whether substances contained in these tissues caused indicator errors when the pH of the lymph was so adjusted that the colors of the tissues were those encountered during life, colors that is to say indicative of reactions toward the alkaline side of the phthalein ranges. A single series of tubes containing the sections immersed in lymph tinted with phthalein was deemed sufficient for this purpose. It was desirable to know more about the liver, kidney, pancreas, and muscle, tissues which appeared to be acid during life, to judge from their hue when stained. For the relative acidity manifested by these tissues might be the result, either of a great indicator error such as would give them the hue of an outspoken acidity when there actually was none, or of a less error that would have the effect of exaggerating a real but slight acidity. Accordingly two sets of tubes were set up, one containing lymph brought to a reaction near the alkaline side of the indicator range, the other with more acid lymph. Shaking with a greater or less quantity of CO_2 in tightly stoppered tubes was the means employed to control the reaction. The colors of the specimens containing phenol red were so adjusted as to give colors indicative of variations from pH 7.0 to 7.40, those with chlor phenol red from pH 6.2 to 6.8, and with brom cresol purple from pH 5.4 to 6.5.

The stoppered tubes, each of which had been colored with 0.08 cc. of a 0.1 per cent solution of indicator, were held at 38°C. for 3 hours on the day of preparation, kept in the ice box overnight, and incubated again for 3 hours. Since the sections appeared but lightly stained, more phthalein was added to the specimens prior to the second incubation, 0.045 cc. to each; and the reactions of many had to be adjusted once again with CO_2. When the tissue colors were to be read the tubes were allowed to cool to room temperature, the colors were read against buffers as in the preceding experiment, and, as in it, the sections were lifted out through paraffin oil for inspection between layers of mica.

The cartilage was not sufficiently stained for comparison with the supernatant fluid. The other tissues submitted to phenol red showed a general correspondence in hue with the surrounding lymph, one notably evident in the case of the colorless tendon, fascia, and pancreatic tissue. A close checking was not possible with slices of muscle, kidney, and liver owing to their natural buffy hue. This much can be said, however, that all of these tissues after exposure to a pink phenol red solution themselves appeared pinkish when flattened out by pressure, not yellow as might have been expected if the yellow coloration observed on vital staining had been due to an indicator error.

The staining with brom cresol purple and chlor phenol red was outspoken, and the colors of the tissues closely corresponded with those of the fluid media both in the slightly and in the more pronouncedly acid series of specimens. For example, the white tendon and fascia exposed to brom cresol purple became blue-green like the lymph about them and so too did sections of pancreas, while other sections exposed to yellow-green lymph became yellow-green. The liver and kidney and muscle exposed to blue lymph (of pH 6.0 to 6.5) became, not blue but

blue-green, owing to the combined buff of the tissue and blue of the phthalein. The fascia and tendon stained with chlor phenol red acquired the pale rose-purple of the lymph from which they were removed; whereas the other tissues, owing to a natural tinting, appeared more or less brownish rose or rosy brown.

Experiments such as the foregoing are of necessity inexact, sharing in this respect with the observations which they were designed to control. The individual findings will not be tabulated, since it suffices to say of them that there was a close correspondence between the tissue hues and those of the enveloping fluid, in despite of an expectation to the contrary based on theoretical grounds. The outcome of the rough tests on the relative accumulation of phthalein upon the tissues under acid and alkaline conditions respectively was in substantial agreement with the previous, more precise, findings at a much higher dilution of phthalein. It showed the slight acidity to have no great favoring influence, under the conditions, upon the phthalein accumulation.

Interpretation of the Dichromatism of Brom Cresol Purple.

Lymph tinted with brom cresol purple and then rendered approximately neutral with CO_2 becomes blue, and, when rendered more acid, a brilliant green when viewed in the test-tube by daylight; and the same is true of tissue extracts in lymph or salt solution acidified with lactic acid, as also of milk, and egg white and gelatin solutions. However the same material when viewed in the colorimeter block does not ordinarily show these anomalous hues, but, instead, the tints resulting from the combinations of orange with purple which constitute a part of the ordinary color range of the indicator. Once only in a large series of instances have we encountered a green hue that made readings impossible. The material was the unusually clear lymph employed in the experiment last described, and it had been saturated with CO_2. When somewhat less CO_2 was employed this lymph appeared blue in daylight but checked well with purple standards in the colorimeter.

Those nearly colorless tissues—connective tissue, cartilage, tendon,

fascia—which appear during life to be almost neutral in reaction[5] are stained blue instead of purple by brom cresol purple injected into a vein or into the peritoneal cavity. When fresh sections of them are rendered faintly acid they take on a yellow-green hue, and the same hue is exhibited by pieces of some of the viscera to which no acid has been added, notably liver, kidney, and pancreas. The green constituent in the color is traceable, like the blue, to the generally recognized dichromatism of the phthalein. We have studied its significance as an indication of the tissue reaction.

The shades of green can be well observed with two-color standards of brom cresol purple when an open rack containing the superimposed tubes is viewed in light from two sources, one passing through them to the eye of the observer, the other striking them sideways. It can then be seen that the color is greenish yellow at pH 5.4, at 5.6 an outspoken green, at 5.8 a purply green, and so too even at pH 6.0. When there is ten times as much phthalein in the standard tubes as Hastings[6] advocates the purple extends much further down the scale, the difference being so marked that it is evident the hue of any given tissue will depend to a considerable extent on the indicator concentration—a fact true of all the phthaleins.

During the addition of lactic acid to tissue extracts for comparative colorimetric and potentiometric reactions with brom cresol purple, as described in our previous paper, the appearance of a green hue was utilized as a sign of developing acidity. But no attempt was made to correlate the various shades of green in the turbid specimens with the electrometric findings, since experience showed that they were not met in the colorimeter, and that excellent readings against buffer standards could be had with this instrument.

There can be no doubt that the series of green hues exhibited by brom cresol purple, under circumstances which bring out its dichromatism, can be correlated, with certain definite reactions, if the phthalein dilution is but known. The green to yellow-green colorations observed in some vitally stained tissues constitute an evidence of acidity as decided as that furnished by phthaleins not subject to dichromatism; and they have this advantage that they cannot be confused with the hues normal to the tissues themselves, as may happen with the colors of phenol red or chlor phenol red when the tinting with these dyes is slight.

[5] Rous, P., *J. Exp. Med.*, 1925, xli, 739.

[6] Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1924, lxi, 695.

Attempts at a Control with Other Indicators.

In the effort to obtain another check on the findings with phthaleins which point to differences in the reaction of individual living tissues,[5] one of us (Drury) has tested out all of the available indicators of Clark's list which are credited with ranges that would fit them for the work. The range of each and its color phenomena were determined with the aid of buffer solutions, and mice were injected with the materials yielding results of promise. Among twenty-six indicators so tested not one was found that lent itself as well to *in vivo* studies as do the phthaleins. Phenacetolin, lacmosol, lacmoid, carminic acid, alizarin sodium sulfonate, and *o*-carboxybenzene-azo-α-naphthylamine failed to stain the tissues well; alizarin V_1, alizarin blue X, and indulin had to be discarded because the ranges differed from those reported, or because the color did not alter notably with slight changes in pH; uranin, dichlorofluorescein, and curcumin manifested confusing fluorescent nuances of hue; monomethyl red, methyl red, ethyl red, and propyl red became fixed on the tissue lipoids; benzene-azo-α-naphthylamine was insoluble in water; and the following indicators stained poorly besides being toxic: *p*-benzenesulfonic acid-azo-α-naphthylamine, naphthylamine brown, aurin, purpurin, alizarin red S, cochineal, cudbear, and brilliant yellow.

Water blue is well tolerated and a vital staining of animals can be accomplished with it; but it is a one-color indicator and the blue varies in intensity not only with the pH but with the amount of the dye present. According to von Möllendorff[7] water blue is gradually reduced to a colorless form within the organism; and we have been unable to demonstrate it in quantity in the pancreas, liver, or kidney, though it is abundant at times in some of the other tissues. When in solution in a concentration that yields a deep blue color at pH 5.29, it is light blue at pH 5.91, and very light blue at pH 6.47, which means that it is ineffective as an indicator in that part of its range which is most important on vital staining. For all these reasons no proper check on the phthalein findings can be expected through its use. The development of acidity post mortem within the subcutaneous tissue can, however, be demonstrated as readily with it as with

[7] von Möllendorff, W., *Z. allg. Physiol.*, 1918, xvii, 125.

phenol red. Animals stained with water blue rapidly become bluer after death. With alizarin sodium sulfonate one can corroborate the observation first made with the aid of phenol red[8] that there occurs an increase in the alkalinity of the subcutaneous tissue of a living animal when this tissue is exposed to air. A reflected skin flap vitally stained with the sulfonate becomes gradually pink during a few minutes of exposure.

Neutral red has been employed by many investigators of the reaction of cytoplasmic granules; and Carnot, Glénard, and Gruzewska[9] have reported upon the differing hue of the various organs which come to contain it. They seem not to have determined whether there was any diffuse coloration. von Möllendorff[10] distrusts neutral red because it is so easily precipitated, with loss of indicator value. Nevertheless the many studies made with this indicator have yielded results in substantial agreement with those obtained through the employment of litmus[1,11] and the phthaleins[5,8] which point to the existence of segregated acid products within mammalian cells of several sorts.

COMMENT.

An essential preamble to any discussion of our findings is a recognition of their limitations. They are the outcome of attempts to determine the influence upon the phthalein colors of tissue materials, not of tissue activities. The problem introduced by these latter must of necessity be begged. It is an axiom with students of vital staining that the relation of living cells to stains is wholly different from that of dead ones. Only in the case of tissues consisting preponderantly of a stainable matrix—connective tissue, cartilage, tendon, fascia, and bone—is there reason to suppose that test-tube phenomena with the phthaleins may approximate those within the living body. The living parenchymatous organs take up or reject the phthalein according to individual peculiarity, and may excrete it in great concentration and

[8] Rous, P., *J. Exp. Med.*, 1925, xli, 451.

[9] Carnot, P., Glénard, R., and Gruzewska, Z., *Compt. rend. Soc. biol.*, 1925, xcii, 865.

[10] von Möllendorff, W., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 2nd edition, 1924, ii, 273.

[11] Rous, P., *Science*, 1924, lx, 363.

into fluids of a reaction markedly different from their own, as is evidenced by the indicator hue.

The color assumed by a living animal injected with a phthalein is due to the extravascular penetration of the dye. Only in proportion as this occurs after an intravenous injection do the tissues color up. By pressure upon those that are accessible *in situ* as for example in the ear, or upon fragments of organs snipped off and placed between mica slides and cover-glasses or between layers of an absorbent material (washed gauze), one can drive out most of the intercellular lymph and demonstrate that the tissues themselves are stained, and not infrequently of a wholly different color from the fluid that had suffused them. They owe their color to phthalein situated within or upon the surface of cytoplasm or matrix. In fresh preparations the cells of bone or cartilage appear as if unstained but ringed in color, either because of a surface deposition, or possibly because they are surrounded with colored lymph; but no such phenomenon is evident with the cells of the parenchymatous organs. The liver, kidney, and pancreas, for example, all show an evenly colored cytoplasm even under a high magnification. The question whether the phthalein is in solution in the cell fluid, or adsorbed, or segregated in finely scattered ultramicroscopic aggregates, cannot at this time be answered. The fact that the indicators are vastly more soluble in water than in fat or ether leaves the point untouched, in our opinion, as for that matter do the results of the tests showing that phthaleins accumulate to but a slight extent upon the water-insoluble elements of dead tissues.

The phthaleins recovered from stained tissues manifest unchanged the color ranges of these substances as originally introduced into the body.[8] The hues of vital stained organs permit of a separation of them into two groups, of those with an apparent reaction slightly less alkaline than that of the blood, and approximating neutrality, and of others with reactions that are to all appearance acid. The findings with the first group might have been predicted *a priori*, for the organs comprised in it have in general few cells, much inert matrix, and a low metabolic activity. One might suppose that the matrix would be a little less alkaline than the blood, as the result of metabolic processes. It is with the second group, of "acid" organs, that interest chiefly lies. Can it be that the hues which these manifest are consequent

not on an actual acidity but upon protein or salt errors, or other errors still, that are referable to the cell materials with which the indicator is associated? The blanket experiments that we have performed yield evidence upon this point. Neither by comparative electrometric and colorimetric tests on extracts of "acid" organs, in water, salt solution, or lymph tinted with indicator,[2] nor by observations on the hue of sections of tissues stained by immersion in phthalein-containing lymph at various reactions have important indicator errors been brought to light. On the contrary the color readings of the reaction prevailing within the tissue extracts and tissue sections have proved unexpectedly dependable. One may urge against the findings with the latter material, to which potentiometer checks were not directly applicable, that the correspondence in color of the sections and the surrounding medium could have been the result merely of an apparent similarity of pH, the reaction of the tissues being in reality not identical with that of the lymph but somewhat different, the difference being concealed through indicator error. It is difficult to believe that so consistent a color approximation could have been attained in this way in all the tests carried out at several reactions with a variety of tissues, and with three indicators.

Many theoretical objections can be made to the use of indicators to determine tissue reactions. Mathews[12] points out that electropositive colloids may unite with acid stains,—and the phthaleins are acid,—with result in a color indicative of acidity when as a matter of fact no acidity exists. von Möllendorff[10] also emphasizes the possibility of indicator error due to the colloids, illustrating it with the example of neutral red. Our experiments show that these sources of error are not important under the conditions with which we have worked. But back of any observations upon living cytoplasm which one may care to make with indicators in general lies the possibility of enormous errors from unforeseen because unapprehended causes. In utilizing these aids to comprehension one enjoys none of the guarantees which go with exact methods and dead matter.

Our electrometric readings on tissue extracts gave practically the same results as those of Michaelis. The extracts of dog liver in water,

[12] Mathews, A. P., Physiological chemistry, New York, 4th edition, 1925, 255.

salt solution, or lymph had reactions ranging from pH 6.34 to 6.73. Michaelis and Kramsztyk,[13] extracting the tissue with several times its volume of water, found a pH of 6.46 in the one instance recorded. With extracts of kidneys prepared in like fashion they obtained for the rat a pH of 6.59 and for the guinea pig other figures between 6.40 and 6.78. Our extract of guinea pig kidney had a pH of about 6.37 and those of dog kidney a reaction ranging from pH 6.39 to pH 6.95. The Kjeldahl figures[2] speak for the presence of tissue derivatives in quantity.

Liver and kidney tissues vitally stained with phthaleins have hues very different from those of extracts of the organs colored with the same indicators, appearing far more acid. To what is the difference attributable? Michaelis and Kramsztyk believed that postmortem changes accounted for the fact that their extracts were faintly acid instead of alkaline like tissue fluids; and to check such processes they boiled some of their specimens, with the result that the pH increased somewhat. The rapidity with which carbon dioxide escapes from tissues had not been fully recognized at the time of their work, and no provision had been made to prevent such an occurrence. A part of the color differences between living and dead tissues stained with phthaleins can be laid to this factor, but one may well doubt whether it is mainly responsible for them. The liver and kidney parenchymas of an animal vitally stained with brom cresol purple or chlor phenol red, when exposed to the air, do not show, like connective tissue, cartilage, and tendon, prompt color changes indicative of a reaction altering toward alkalinity as they inevitably would were carbon dioxide the cause of their acidity.

Michaelis' figures for the pH of tissue extracts have been largely accepted as representative of the reactions prevailing within the living body. Only recently Haldane has invoked them[14] in justification of an hypothesis whereby the observed facts as to the permeability of cells to electrolytes are made to account quantitatively for the hydrogen ion concentrations of the tissues.

[13] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

[14] Haldane, J. B. S., *Proc. Cambridge Phil. Soc. Biol. Sc.*, 1923–25, i, 243.

SUMMARY.

The influence of lymph-insoluble tissue materials upon the colors manifested by phthalein indicators has been tested by comparing the hue of sections of various organs, stained by immersion in colored lymph, with the hue of the surrounding fluid. The reaction has been brought to approximate that of life by a greater or less saturation of the material with carbon dioxide. No evidence has been obtained of indicator errors referable to the association of the phthaleins with tissue substances.

The inferences to be derived from the experiments, and from others detailed in a preceding paper, are discussed. The results of them all are in essential agreement. They demonstrate that the observed hues in tissues vitally stained with phenol red, brom cresol purple, and chlor phenol red cannot be laid to indicator errors resulting from an association of the phthalein with tissue materials. The fact is of special note in connection with organs which exhibit, when stained, the colors indicative of an outspoken acidity.

The findings constitute a control upon the influence of the tissue materials on the colors manifested by the phthalein indicators when used as vital stains; but they leave untouched the problem of the influence of tissue activities upon the coloration.

STUDIES OF HYPERTHYROIDISM.

III. Bile Pigment Production and Erythrocyte Destruction in Thyroid-Treated Amphibian Larvæ.

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Plate 26.

(Received for publication, February 24, 1926.)

Many profound changes, internal as well as external, occur rapidly in amphibian larvæ in which an experimental hyperthyroid condition is induced. During the progress of experiments upon thyroid-fed tadpoles pronounced variations became apparent in the color of the bile. These variations, moreover, were quite regular. Accordingly, special attention was directed to the gall bladder and liver with the object of obtaining data in regard to the change in bile pigment production and the possible significance of this change as an indicator of erythrocyte destruction.

The material used consisted of larvæ of the tree frogs (*Hyla crucifer* and *Hyla versicolor*), of the green frog (*Rana clamitans*), of the toad (*Bufo americanus*), and of the salamander (*Amblystoma punctatum*). These were collected at Woods Hole, Massachusetts, during the latter part of June and early July. Additional green frog tadpoles were collected during the winter at Charlottesville, Virginia. In age and stage of development the animals varied greatly, ranging from 2 weeks after hatching, as in the case of the toad tadpoles, to near the time for metamorphosis, as in some of the tree frog tadpoles. Well developed hind limbs, but no erupted fore limbs, were present in the tadpoles of *Hyla crucifer*, *Hyla versicolor*, and *Rana clamitans*. In one batch of *Hyla crucifer* tadpoles the hind limbs were scarcely visible. In the toad tadpoles the hind limbs were not visible. The salamander larvæ possessed prominent external gills, and both limbs were well developed. Metamorphosis was accelerated and the hyperthyroid condition induced by placing dried sheep thyroid extract in the water with the animals. In a few cases pure thyroxin crystals were used. Animals were killed and the gall

bladders examined at intervals of 1 to 21 days. A few animals were killed and examined during the first 24 hours of treatment. All parts of the body were examined microscopically and a detailed study was made of the liver, spleen, kidney, gut, gills, and tail with particular attention to the conditions of erythrocyte destruction. The Giemsa eosin-azure blood technique was employed. Observations were also made on preparations of living blood.

OBSERVATIONS.

The macroscopic changes observed in *Hyla crucifer* are clear-cut. They are also similar to those observed in the other species studied and may be taken as typical. The normal gall bladder full of bile is pale watery green and quite transparent. The bile itself, when made to stain a piece of white paper, presents a very faint tinge of green. At the end of the 1st day of thyroid treatment a very slight deepening of the green color may sometimes be detected. At the end of the 2nd day the color is definitely a deeper green and this becomes progressively more pronounced in 3, 4, 5, and 6 day old thyroid-treated animals. The normal pale green color of the gall bladder changes to a deeper brighter green, then to an emerald-green, and finally to a dark emerald-green. The bile pigment, biliverdin, apparently becomes increasingly concentrated in the gall bladder. During this period the animal exhibits the typical changes of metamorphosis, being distinctly slimmer and smaller at the end of 2 days, the fore limbs erupting after about 4 days, the gills and tail degenerating, the latter being nearly resorbed at the end of 6 days. In the case of the smaller and more immature animals, metamorphosis proceeds only to a certain critical stage. Metamorphic stasis results, usually followed by death.

In the case of the larvæ of the tree frog (*Hyla versicolor*) the color of the normal full gall bladder is a pale transparent greenish yellow with perhaps more yellow than green. With thyroid treatment the green becomes much deeper. In the case of the green frog tadpole some yellow is also present in the bile. This tadpole, because of its large size, is not so quickly affected by the thyroid administration. The succession of color changes presented by the bile proceeds somewhat more slowly. The larger the animal the longer the latent period required for the thyroid extract to produce its effect. Thus, in this species the bile color changes are most pronounced after from 4 to 20 days following the first thyroid administration. In salamander

larvæ the changes from very pale green to deep green could best be seen after from 4 to 15 days of treatment with thyroid extract.

In all species it was possible to arrange a color series with the pale green, or pale yellowish green normal gall bladders at one end and the dark green of the advanced thyroid-treated animals at the other end, with the intermediate shades of green between, varying in depth according to the length of thyroid treatment. A few observations upon untreated tadpoles undergoing normal metamorphosis show that there is a deepening of bile color. Although quite definite, this is not so pronounced as in the experimental tadpoles undergoing thyroid-accelerated metamorphosis.

TABLE I.

Species.	No. of normal animals.	No. of thyroid-treated animals.
Hyla crucifer	20	20
Hyla versicolor	38	16
Rana clamitans	21	15
Amblystoma punctatum	17	14
Bufo americanus (2 wks. after hatching)	250	300
Bufo americanus (older)	15	40
Total	361	405

Table I shows the number of observations made upon normal and experimental animals in each species.

All normal immature tadpoles possessed pale colored bile with relatively little pigment concentration. All thyroid-treated animals after a latent period of about 2 days exhibited darker colored bile with very marked increase in pigment concentration. No exceptions were seen.

Histological Data.

The Liver.—Some increase in erythrocyte degeneration was evident in the sinuses of the liver in the case of the thyroid-treated animals. This was apparent from the increased number of fragments of erythrocytes and abnormal forms (Figs. 1 to 4 and 7). The erythrocyte fragments often become aggregated in groups, with a tendency for the

fragments to round up into globules. In the later stages of thyroid treatment the number of large mononuclear phagocytic cells (macrophages, monocytes, reticulo-endothelial cells) with ingested erythrocyte débris becomes markedly increased (Figs. 5 and 6). Melanin-like pigment is also prominent in the liver, being handled by the macrophages and often being associated in these cells with hemoglobin débris from worn out erythrocytes.

The Spleen.—The activity of the spleen seems to vary greatly as regards erythrocyte degeneration in different individuals. The histological evidence indicates that there may be some increase in erythrocyte destruction by fragmentation (Figs. 8 and 9). Typical macrophages are present containing ingested erythrocyte fragments and globules (Fig. 10), but a definite increase in the number of these cells in the spleen was not seen. The fact must be taken into account, however, that the sinuses of the spleen are largely drained of their cellular content after the thyroid extract has exerted its effect (1).

The Kidney.—There is almost no erythrocyte destruction in the kidney either of the normal or of the thyroid-treated tadpole.

The Gut.—In many regions throughout the gut of thyroid-treated animals from pharynx to lower intestine, there may be seen many macrophages passing through the lining toward the lumen (Fig. 12). These are especially conspicuous during the later stages of thyroid administration. They contain erythrocyte débris largely. Very active sloughing off of lining cells takes place, and associated with this is a certain amount of extravasation of erythrocytes.

The Gills.—In the degenerating gills of the later thyroid-treated animals may be seen many macrophages filled with ingested erythrocyte fragments (Fig. 11). These seem in many cases to pass through the gill epithelium into the gill chamber, and are thus lost to the body.

The Tail.—In the degenerating tail of thyroid-treated tadpoles during the process of resorption may be seen extravasations of red blood cells.

Fresh Blood Preparations.—Fresh blood mounts were made and examined, the cover-glass being sealed with vaseline. More non-nucleated erythrocyte fragments were apparent in the thyroid-treated tadpoles, both in the liver blood and in the peripheral blood.

INTERPRETATION AND DISCUSSION.

The observations upon the bile color of normal and thyroid-treated tadpoles leaves no doubt but that the hyperthyroid condition brings about greatly increased bile pigment concentration in the gall bladder. Such concentration might be favored by (1) a decrease in the amount of water passed into the bile; (2) resorption of water from the gall bladder; (3) an increase in the rate of bile pigment formation. Each of these possibilities may be considered.

1. It is known that water is extruded from the body of a tadpole during thyroid-accelerated metamorphosis. The tissues are dehydrated to a certain extent. Loss of water from the tissues of the animal as a whole might be reflected in a decreased amount being passed into the bile. To a limited extent, this may account for the deeper bile color that does occur.

2. There is a possibility that water may be resorbed from the gall bladder bile, especially if the bladder becomes somewhat distended. During the first couple of days of thyroid treatment the gut is emptied of its contents, and it then remains during the metamorphosis practically devoid of food. The animal eats little or nothing during this period. With the stimulus of food in the alimentary tube lacking there may be a decrease in the amount of bile passing from the gall bladder to the intestine. This may lead to an accumulation of bile in the gall bladder. If water is then resorbed the bile pigment would become more concentrated. There is no special evidence that this process does take place, but it may be a minor factor in bringing about the pigment concentration in the gall bladder. It hardly seems entirely adequate, however, to account for the extreme changes observed.

3. In all probability the major factor contributing to pigment concentration in the gall bladder bile is a greatly increased rate of elaboration of the pigment in the thyroid-treated animals. The bile pigments, biliverdin and bilirubin (and derivatives), are believed to be formed from hemoglobin. In Rich's recent review of the bile pigment situation (2), the conclusion is reached that hemoglobin furnishes the chief and perhaps the only source of bile pigment. The supply of hemoglobin must come from worn out erythrocytes. There is abundant evidence that erythrocyte destruction proceeds at a greatly accelerated rate in the thyroid-fed tadpoles. This destruction precedes

and accompanies the increased rate of bile pigment production. Strong support is, therefore, afforded for the deduction that this constitutes the chief part of the mechanism by which hyperthyroidism brings about the characteristic color changes in the bile.

That thyroid autacoid should have a destructive effect upon the erythrocytes is not surprising. It is rather to be expected. A rapid change from the relatively tranquil life of the tadpole with low metabolic rate to a quickened existence with high metabolic rate is induced by the thyroid administration. Helff (3) has shown that the oxygen-carbon dioxide exchange is greatly heightened. The circulation is also speeded up. The importance of the erythrocyte in relation to these changes is apparent. The senile erythrocytes are ready for destruction at a greatly increased rate. At the same time large numbers of lymphoid hemoblasts are stimulated to proliferate and to undergo differentiation into young erythrocytes. It is characteristic of thyroid treatment that it effects a rejuvenation process; first, by destruction or elimination of the older and more highly differentiated cells, and second, by stimulation of undifferentiated cells to proliferate and then to differentiate. The destruction of the older erythrocytes represents the first step in the blood cell reorganization. The second step, the stimulation of the undifferentiated bood cells, the hemoblasts and their mesenchymal precursors, has already been considered in detail in an earlier contribution (1). A parallel example is found in the response of epithelial surfaces to thyroid administration. In the skin the outer older layers are sloughed off and eliminated. The basement membrane layer containing the younger less differentiated cells is stimulated to proliferate and produce new cells, which later become more highly differentiated. The same activities are exhibited by the lining of the gut throughout a large part of its extent. The blood cells, therefore, in responding to thyroid administration by destruction of the older types (senile erythrocytes) and by stimulation of the younger types (lymphoid hemoblasts and their mesenchymal predecessors) are not unique, but are like other cells of the body.

The senile erythrocytes often seem to lose their nucleus before fragmenting. This may occur by nuclear extrusion, nuclear resorption, or cytoplasmic segmentation (Figs. 3, 4, 7, 8, and 9). This condition in the frog tadpole is of interest for comparison with the con-

ditions in urodeles. Emmel (4) has pointed out that in many urodele amphibians a small percentage of the red blood cells is normally without a nucleus. These arise by "cytoplasmic segmentation" from the nucleated erythrocytes.

In the frog tadpole the senile cells and fragments are rapidly filtered out from the general circulation, chiefly in the liver and spleen. Here fragmentation and globule formation proceed, followed by ingestion by the macrophages. Phagocytosis of erythrocytic globules and fragments becomes more conspicuous in the later stages of thyroid treatment. Groups of macrophages may finally undergo extensive disintegration, especially in the liver and spleen. Although these cells are probably of importance in the elaboration of the bile pigment, the exact mechanism of the process is not known.

The appearance of the gut is of special interest. Macrophages containing ingested material, some of which is erythrocyte débris, pass in large numbers through the lining of the gut into the lumen (Fig. 12). Presumably they are then egested with the intestinal contents. It is, of course, possible that some of this material may be reabsorbed. Likewise, in the atrophying gill region macrophages containing erythrocyte débris apparently pass through the gill epithelium to the gill chamber and thus leave the body (Fig. 11). This apparent loss to the body of destroyed erythrocytes suggests the conclusion that no special attempt is made to conserve the hemoglobin.

Rous and Robertson (5, 6) in mammals of several species have shown the importance of the method of red blood cell destruction by fragmentation, both in normal animals and in animals rendered either anemic or plethoric. Essentially similar conditions appear to obtain in normal and thyroid-treated tadpoles. Rous and Robertson (6) also find that in animals rendered plethoric or anemic increased destruction of red corpuscles by fragmentation takes place without loss of hemoglobin. In the anemic animals there is no increase in bile pigment output. These results are of interest for comparison with the conditions in thyroid-treated tadpoles. In the tadpoles hemoglobin is excreted at a greatly increased rate, both in the form of bile pigment and in the form of hemoglobin débris contained within the macrophages which migrate through the lining of the gut and through the epithelium of the involuting gills. As the gut wall is itself undergoing

profound involution changes, it is likely that little or no reabsorption of this material occurs. The whole reaction is probably a vicious one, as far as the best interests of the tadpole are concerned. In spite of the fact that thyroid treatment induces a higher metabolic rate, which would seem to require a greater hemoglobin basis for its maintenance, the body apparently gets rid of a part of its hemoglobin very rapidly. Thus it is that the anemic condition results, which is associated with metamorphic stasis, and which may be partly the cause of the usual death in this stage.

Rous and Robertson regard it as probable that the normal fragmentation of the red cells may be a result of the perpetual sieving and squeezing to which they are subjected in the finer capillaries. Under conditions of vigorous exercise Broun (7) has shown that increased blood cell destruction occurs, and Broun, McMaster, and Rous (8) have shown that increased bile pigment output results. In the case of the tadpoles, thyroid administration is followed by increased rate of heart beat, increased circulatory rate, and increased muscular activity. It is clear, therefore, that under these conditions the erythrocytes would be subjected to rougher treatment than under normal conditions. In this way the increase in erythrocyte destruction and fragmentation can be accounted for.

The possible influence of food on bile pigment formation, such as has been claimed by Whipple and Hooper (9), may be ruled out in the experiments on thyroid-treated tadpoles. Animals undergoing rapid thyroid-induced metamorphosis eat practically nothing. Usually they were kept in jars containing no food material except that put up with the thyroid extract. Furthermore, the typical changes occur in animals after the administration of pure thyroxin crystals only.

SUMMARY.

Experimental hyperthyroidism in urodele larvæ (*Amblystoma*) and anuran larvæ (*Rana*, *Bufo*, and *Hyla*) is accompanied by definite changes in bile color. The normal pale green, or pale yellow-green, color of the full gall bladder changes progressively after thyroid administration to a brighter green, then emerald-green, and finally a very dark green. In several hundred observations no exceptions were noted.

The bile pigment, biliverdin (and its derivatives), is elaborated from the hemoglobin of worn out erythrocytes. Thyroid administration induces an increased rate of erythrocyte destruction, and this is followed by an increased output of bile pigment. Other minor factors are mentioned which may to a limited extent modify the color of the bile.

Erythrocyte destruction occurs largely by enucleation, cytoplasmic segmentation, and fragmentation, and is probably widespread in the body. Many fragments and senile red cells collect in the liver. During the later stages of thyroid treatment the macrophages become conspicuously active. They are especially abundant in the liver, the gut, and the gills. In addition to the hemoglobin eliminated after transformation into bile pigment, some is transported by macrophages through the gut lining, and to a less extent through the involuting gill epithelium, and thus eliminated from the body.

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[1] This paper gives an extensive bibliography on the subject of bile pigment.

EXPLANATION OF PLATE 26.

All drawings were made from tissues of a green frog tadpole (*Rana clamitans*). This specimen was killed 15 days after the first thyroid administration and shows all of the typical stages in erythrocyte destruction and removal. The tissues were stained with the Giemsa eosin-azure mixture.

FIG. 1. Two normal erythrocytes. × about 1045.

FIG. 2. Degenerating globules of worn out erythrocytes from liver sinus. × about 1045.

FIG. 3. Two large erythrocyte fragments from liver sinus. × about 1045.

FIG. 4. Several erythrocytic globular fragments and two erythrocytes after nuclear extrusion or resorption, from liver sinus. × about 1045.

FIG. 5. Large mononuclear phagocyte (macrophage, monocyte, reticulo-endothelial cell) from liver sinus, containing one large erythrocyte fragment and many smaller ones. × about 1045.

FIG. 6. Large mononuclear phagocyte from liver sinus, with two ingested hemoglobin-containing globules and a few granules of a melanin-like pigment. × about 1045.

FIG. 7. Erythrocyte from liver sinus apparently about to undergo enucleation. × about 1045.

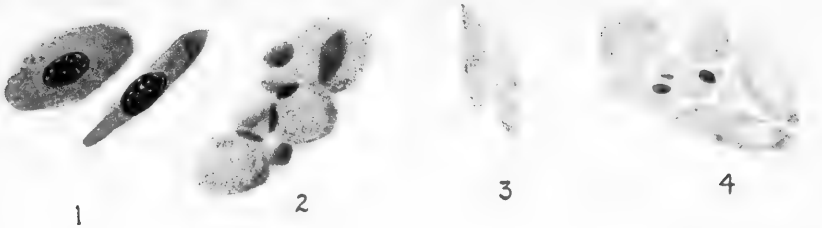
FIG. 8. Enucleated erythrocyte fragment from the spleen. × about 1045.

FIG. 9. Two nucleated erythrocytes from the spleen whose contour suggests one method of globule formation by cytoplasmic segmentation. The second cell from the left represents an enucleated erythrocyte with its cytoplasm at one pole fragmenting into globules. At the extreme right are three hemoglobin-containing globules, possibly derived from a single senile erythrocyte. × about 1045.

FIG. 10. Macrophage from the spleen containing two large and two small erythrocytic globules. × about 1045.

FIG. 11. Cross-section through a gill filament showing three macrophages (*m*) loaded with erythrocyte débris, passing through the gill epithelium (*e*). × about 460.

FIG. 12. Section through the posterior portion of the intestine, showing groups of macrophages (*m*) migrating through the lining (*l*). The macrophages contain other ingested material in addition to the erythrocyte débris. × about 460.



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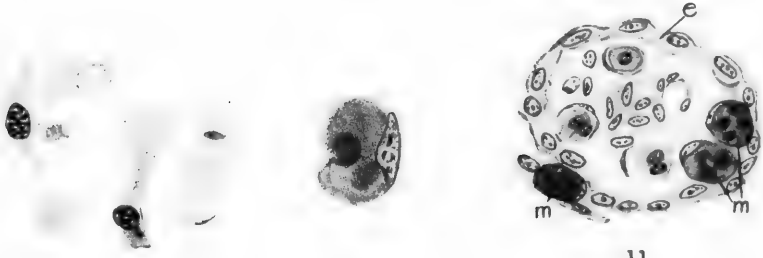

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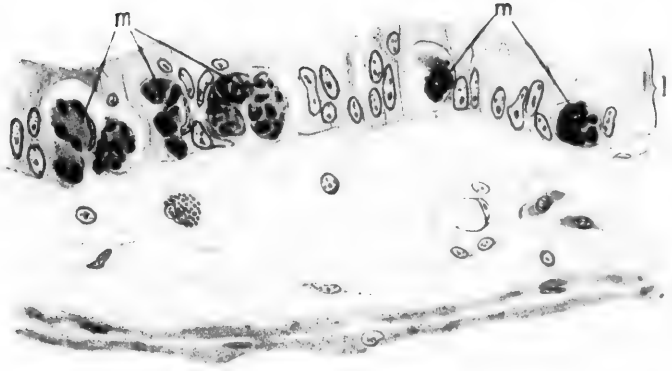

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(Speidel: Hyperthyroidism. III.)

UNIV. OF MICH.

CHANGES IN THE REACTION POTENTIAL OF A TRANSPLANTABLE TUMOR.*

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PLATE 27.

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INTRODUCTION.

In the present paper a tumor will be described which gave very uniform results in a series of mice of known pedigree for a period of about a year. We may say that the reaction potential existing between the tumor and the individuals inoculated was constant. In the course of routine transplantation of the tumor, there was a sudden change in this hitherto constant reaction potential resulting in at least three new types of transplantable tumors—new types as far as their proliferative vigor and reaction potential (transplantability) are concerned.

It is the purpose of this paper to discuss the bearing of the phenomenon of the change in the reaction potential of the transplantable tumor on the so called genetic theory of transplantation, together with some remarks concerning the problem of tissue specificity and allied topics.

The Tumor.

The tumor which has been employed in the present investigation is the fourth spontaneous neoplasm to arise in a relatively homogeneous dilute brown strain of mice which has been rigidly inbred brother to sister for at least 40 generations. The other three tumors, which were designated dBrA, dBrB, and dBrC, were subjects of extensive experimental studies to determine the influence of the genetic make-up of the tumor and of the host on the fate of transplants.[1,2] It was found

* This experiment has been made possible by a grant from The Rockefeller Institute for Medical Research.

[1] Little, C. C., and Strong, L. C., *J. Exp. Zool.*, 1924–25, xli, 93.

[2] Strong, L. C., *Genetics*, in press.

that all animals of the dilute brown strain were uniformly susceptible to all three tumors. On the other hand, another inbred strain, known as the Bagg albino strain, was found to be uniformly resistant to the same transplantable tissue. Further analysis showed that the first filial generation of mice resulting from crossing the original dilute brown strain with the Bagg albinos was uniformly susceptible to all three tumors. In the second filial generation, two classes of individuals were obtained, the relative percentage of susceptible to non-susceptible individuals being different in each of the three neoplastic tissues employed. These findings were interpreted as indicating that Mendelian factors were involved in the transplantation of malignant tissue.

From these experiments we developed the so called genetic theory of transplantation, which may be stated as follows: susceptibility to a transplantable neoplastic tissue is brought about by a reaction between the host and the tumor cell. The reaction of the host is governed largely by its genetic constitution (derived from the zygote from which it arose) and that of the transplant is somewhat controlled by certain intrinsic or genetic factors.[1]

The new tumor arising in an individual of the same dilute brown strain of mice we have designated dBrD, in order to continue the nomenclature previously used. It is a mammary gland carcinoma. A photomicrograph of the tumor is given in Fig. 1.

EXPERIMENTAL.

Experiment 1.—The first experiment consisted in the inoculation of the new tumor, dBrD, into individuals of the inbred dilute brown strain. It was found that they were uniformly susceptible to the transplant (94.00+ : 0.00− or 100.00 per cent susceptible). At the same time another series of individuals belonging to the Bagg albino strain was inoculated with some of the same transplantable tumor. It was determined that these individuals were uniformly resistant (0.00+ : 104.00− or 0.00 per cent susceptible). The F_1 generation produced by crossing these two stocks gave individuals all of which grew the transplant (125.00+ : 0.00− or 100.00 per cent susceptible). It is evident therefore that the new dBrD tumor gave very similar results to the three previous tumors of the same series, dBrA, dBrB, and dBrC. At the present time 112 individuals from the F_2 generation have been inoculated with the transplantable tumor, dBrD, and 16 of these grew the transplant progressively, while 96 proved to be resistant to the same tissue.

In attempting to fit the observed data to Mendelian principles as has already been so successfully done in the case of five transplantable tumors (two by Tyzzer and Little, two by Little and Strong, and one by Strong), it is evident that the behavior of the transplant placed it in the

same class as the three other transplantable tumors derived from the same inbred dilute brown stock of mice. The results can be explained if the assumption be made that for the successful growth of the transplant there must be present within the host at least from six to eight independently inherited genetic units or factors (genes). It is probable that the number of genetic factors is seven. The degree of probability determined by the comparison of the probable error between observation and expectation for six, seven, and eight factors is greater in the case of seven factors than it is for the other two possibilities although the six and the eight factor interpretations are not definitely excluded by the present data.

The degree of significance between the observation on the original dBrD tumor and the expectation in the F_2 generation according to Mendelian principles is given in Table I.

After the collection of the above data, no further genetic studies being contemplated, the tumor was simply continued for some generations in individuals of the F_2 generation. In the course of this routine continuation of the tumor, it was found that one of the transplants grew with remarkable rapidity. In subtransplants from this particular tumor the rapidity of growth continued. This phenomenon had already been encountered in the case of the dBrC tumor.[2] I had also already determined that the average rate at which a transplant grew within a given constant genetic strain of mice was an index of its intrinsic or genetic make-up. I consequently continued the transplantation of this aberrant type. Two other subtransplants were observed that apparently had deviated from the normal growth rate of the original dBrD tumor. These transplants were also continued.

Experiment 2.—The second experiment consisted in the inoculation of the first aberrant tissue, which recived the name dBrDm, into a number of F_2 individuals. It was found that of the 99 individuals inoculated, 56 grew the transplant progressively while 43 proved to be resistant to the same tissue. The data are tabulated in equation 5, Table II.

Experiment 3.—Another series of F_2 individuals was inoculated with the second aberrant tissue, dBrDBl. 122 individuals of this generation were inoculated. Of these, 66 were susceptible and 56 were negative. The data for this neoplastic tissue are given in equation 9, Table II.

Experiment 4.—The third tissue, dBrDBs, was inoculated into a number of F_2 individuals. In this case 124 individuals were employed. 88 of these were sus-

TABLE I.

Comparison of the Observed Data for the F_2 Generation Together with the Expectation for the Six, Seven, and Eight Mendelian Factors. Original dBrD Tumor.

	F_2 generation.	No. of individuals.	Per cent negatives.	Difference between.
1	Observation.	16.00+:96.00−±2.47	85.71%±2.20	
2	Expectation 6 factors.	19.96+:92.04−±2.70	82.14%±2.41	1 and 2 = 3.57%±3.26 or 1.09 × *PE.*
3	" 7 "	14.95+:97.04−±2.40	86.61%±2.18	1 " 3 = 0.90%±3.11 " 0.03 × *PE.*
4	" 8 "	10.20+:101.80−±2.03	90.89%±1.82	1 " 4 = 5.18%± 2.85 " 1.81 × *PE.*

TABLE II.

The Data Obtained for the Three Transplantable Tumors, dBrDm, dBrDBl, and dBrDBs, in the F_2 Generation Together with the Comparison for Similar Data Obtained with the Original dBrD Tumor.

Experiment No.	Name of tumor.	Equation.	F_2 generation.	No. of individuals.	Per cent negatives.	Difference between equations.
1	Original dBrD	1	Observation.	16.00+:96.00−±2.47	85.71%±2.20	
2	dBrDm	5	"	56.00+:43.00−±3.29	43.43%±3.32	1 and 5 = 42.28%±3.98 or 10.62 × *PE.*
		6	Expectation 1 factor.	74.25+:24.75−±2.91	25.00%±2.94	5 " 6 = 18.43%±4.52 " 4.07 × *PE.*
		7	" 2 factors.	56.68+:43.31−±3.28	43.75%±3.31	5 " 7 = 0.32%±4.88 " 0.09 × *PE.*
		8	" 3 "	41.77+:57.23−±3.27	57.80%±2.88	5 " 8 = 14.37%±3.80 " 3.78 × *PE.*
3	dBrDBl	9	Observation.	66.00+:56.00−±3.66	45.90%±3.00	1 " 9 = 39.81%±3.72 " 10.70 × *PE.*
		10	Expectation 1 factor.	91.50+:30.50−±3.19	25.00%±2.61	9 " 10 = 20.90%±3.97 " 5.01 × *PE.*
		11	" 2 factors.	68.63+:53.37−±3.65	43.75%±2.99	9 " 11 = 2.15%±4.24 " 0.51 × *PE.*
		12	" 3 "	51.48+:70.52−±3.63	57.80%±2.98	9 " 12 = 11.90%±4.22 " 2.82 × *PE.*
4	dBrDBs	13	Observation.	88.00+:36.00−±3.03	29.03%±2.44	1 " 13 = 56.68%±3.28 " 17.28 × *PE.*
		14	Expectation 1 factor.	93.00+:31.00−±3.21	25.00%±2.42	13 " 14 = 4.03%±3.43 " 1.17 × *PE.*
		15	" 2 factors.	69.75+:54.25−±3.71	43.75%±3.00	13 " 15 = 14.72%±3.87 " 3.80 × *PE.*

ceptible while 36 failed to show progressively growing masses. These data are tabulated in equation 13 of Table II.

It may be well to point out, however, that some of the F_2 individuals were inoculated simultaneously with two of the tissues, so as to compare the tissues in the same soil. When this was done, one of the tumors was placed subcutaneously in the right axilla, while the other one was placed in the left axilla.

The data obtained in Experiments 2, 3, and 4 for these new transplantable tumors, in the F_2 generation, together with the comparison for similar data obtained with the original dBrD tumor (Experiment 1), are given in Table II. The degree of significance, based upon the calculation of the probable error, is also given. For every tumor, the data are given for the estimated nearest Mendelian interpretation or expectation.

The original dBrD tumor failed to grow in 85.71 per cent of the second filial generation individuals. It may be said, therefore, that the tumor is highly specific, or that it possesses a high degree of tissue specificity. Very few F_2 individuals have the necessary make-up for the continued growth of the transplant. From this high state of specificity, there split off during the course of the present experiment new tissues that gave different degrees of specificity (transplantability) in the same series of F_2 individuals; one subtransplant, dBrDm, growing in all but 43.43 per cent of the individuals; another, dBrDBl, giving 45.90 per cent negative reactions; and a third, dBrDBs, growing in all but 29.03 per cent of the F_2 individuals. The degree of specificity for two of the subtransplants, dBrDm 43.43 per cent and dBrDBl 45.90 per cent, is about the same. Experiments are now under way to ascertain if this result is determined by the same genetic background or whether a similar genetic complex is involved.

The degree of probability of difference between the original dBrD and the three subtransplants is significant in every case; that degree of probability difference between the first subtransplant dBrDm and the original dBrD tumor is 10.62 times the probable error; the degree of probability difference between the second subtransplant dBrDBl and the original dBrD tumor is 10.70 times the probable error; while in the case of the third subtransplant, dBrDBs, there is 17.28 times the probable error difference.

Obviously there must have been clear-cut variations from the type reaction which had been investigated for over a year. From a state of high specificity, the transplantable tumor has deviated into other types showing different degrees of specificity. Correlated with this change of tissue specificity there was also obtained an increased growth or proliferative vigor. The data on the growth rates are reserved for publication in the near future.

Photomicrographs of tumors dBrDm and dBrDBs are given in Figs. 2 and 3.

SUMMARY.

A transplantable tumor, known as dBrD, gave very uniform results in a series of mice of known pedigree for a period of about a year.

TABLE III.

Tabulated Synopsis of the Various Transplantable Tumors Used in the Present Experiment, Showing the Probable Number of Independently Segregating Genetic Factors Involved in the Process of Transplantation.

Name of tumor.	No. of genetic factors.	No. of F_2 individuals used.
Original dBrD	7	126
dBrDm	2	99
dBrDBl	2	122
dBrDBs	1	124

We may say that the reaction potential existing between the tumor and individuals inoculated was the same during this first part of the experiment. This tumor will grow in a mouse progressively provided there be present simultaneously at least from six to eight independently segregating genetic factors (genes) in the fundamental make-up of the host derived from the zygote that gave rise to the individual. In the course of routine transplantation of this tumor, there was a sudden change in this hitherto constant reaction potential resulting in at least three types of transplantable tumors—new types as far as their physiological activity and reaction potential are concerned. From a single original transplantable mass, I have therefore obtained four masses that remain true to type at least for some months of continued experimentation. These four types are (1) the original tumor,

dBrD, still giving a six to eight Mendelian factor ratio, (2) dBrDm giving a two factor ratio, (3) dBrDBl showing another two factor ratio, and (4) dBrDBs giving a one factor ratio. The number of factors involved in the transplantability for the several tumors investigated are given in Table III.

GENERAL DISCUSSION.

It is probable that neither the theory of adaptation nor the theory of virulence will explain completely the data outlined in the present paper. These theories are descriptive phrases applied to the tumor mass and apparently enter into the equation after the change or changes have been produced; that is, they are the result of the change and not the cause of the phenomenon.

If the genetic theory for the transplantation of malignant tissue is valid, then it ought to demonstrate a mechanism whereby such a change in the reaction potential of the transplantable tumor, as described in this paper, may be brought about. The change, whatever it is, must of course be either in the host component or in a possible tumor variable. Let us consider first the host. Obviously, if the host's tolerance of the transplantable tumor is dependent to a large extent at least upon its genetic constitution, when a change occurs in the genetic constitution of the host then this change of necessity would alter the reaction potential under consideration. Changes in the genetic constitution of the mice employed, however, cannot explain the present data. The second filial generation individuals which gave the indication of the genetic complex involved, as far as the host was concerned, had been picked out at random and inoculated with one type of transplantable tumor or another. The parents were the same for all the F_2's, while the number of susceptible grandparents was very small. Sometimes a single mouse was inoculated with two different types of tumors simultaneously. The genetic constitution of the mouse, as far as this experiment on testing its physiological response to two different types of tumors was concerned could not possibly have been different. For these reasons, the host complex during this experiment must have been a constant one, or at least, has not influenced the observed change in the reaction potential.

Several suggestions arise as to how this change in the reaction poten-

tial may be brought about by a change in the tumor component. The most obvious one is that perhaps a new neoplasm has developed out of the host tissue in contact with the malignant tissue inoculated, either as a matter of chance or as a result of some influence exerted by the transplant upon the host's cells in that locality. There are several reasons that appear to throw considerable doubt on this conception. First, the histological appearance of all the subtransplants is very nearly the same as that of the original tissue (Figs. 1 to 3). Second, the F_2 generation is one in which the range of variability among the individuals is enormous. It is extremely difficult to get a spontaneous tumor from any F_2 individual to grow in any other individual. Yet in this experiment the subtransplants grew in an increased number of F_2 individuals. (Spontaneous tumors arising in F_2 individuals have been found to be, without exception, highly specific, whereas the subtransplants encountered in this experiment show low specificities.) Third, all individuals in this experiment were inoculated at 6 weeks of age. The incidence of spontaneous carcinoma in individuals of this very immature age is certainly of rare occurrence. Fourth, the change from high specificity toward a lower degree of specificity is a phenomenon of rather frequent occurrence. I have, however, never encountered any change in the opposite direction, although I have watched and devised experiments to detect such a change if it ever occurred. For these four reasons, primarily, the probability of a new spontaneous tumor arising in the inoculated individual is extremely slight.

Another possibility is that by some fortunate circumstance the tumor was a mixed tumor to begin with, that is that the mass was made up of a mixed aggregate of cells of differing potentialities—the less malignant strains were killed off to the benefit of the more malignant ones. There appear to be pretty good grounds for considering the majority of spontaneous tumors as mixtures, and transplanting them to many diverse soils cannot fail to result in selection. By continued transplantation the less adaptable tissue is weeded out and the tumor gains "virulence" and after a time tends to a more fixed type. This suggestion is tenable but is not satisfactory. Since all the derived transplants were made up of histologically indistinguishable cells, one would have to assume a mixture of at least four histologically identical

cells all possessing different physiological characteristics. The nature and origin of such a mass could only be conjectured—not demonstrated nor proven. There are several difficulties in the way of accepting this interpretation. First, the phenomena of the change in the reaction potential has been encountered several times. In fact I have witnessed it in seven of the nine neoplastic tissues derived from individuals of this inbred dilute brown strain of mice. In every case, this breaking down in the tissue specificity of the tumor mass has occurred after the mass had settled down to a *constant* type for at least several months. Second, at every inoculation the tumor was pulped and pushed through a small trochar into the mice inoculated. The tumor is rather a soft one. There is therefore only a slight possibility of all *four* types of cells clinging together at every inoculation for more than a year—and then all separating from each other within a period of 3 weeks. The number of neoplastic cells that survive after transplantation is certainly very limited, thus militating against the persistence of four distinct types of cells during the extent of the first part of the experiment in which the reaction potential of the mass remained constant. Third, the proliferative vigor of the various types of cells differed considerably. The subtransplants showing a low degree of tissue specificity possess several times as much proliferative vigor as those showing high specificity. The slow growing cells would consequently have been completely swamped in a period of a year. Both Ehrlich[3] and Apolant[4] determined that "synthetic" mixed tumors could *not* be produced by the intermingling of two distinct types of neoplastic tissues, provided the vitality and proliferative energy of the two components differed to any marked degree.

For these several reasons, the probability is that the tumor was not a physiologically mixed tumor at least at the time of the occurrence of the change of the reaction potential, although this conception is not absolutely ruled out by the present data.

Another conception is that this change of the reaction potential existing between the tumor and the host inoculated is brought about by an internal change in the genetic constitution of the tumor cell. This hypothesis of course implies that the tumor cell is controlled to

[3] Ehrlich, *Z. Krebsforsch.*, 1907, v, 67.

[4] Apolant, H., *Z. Krebsforsch.*, 1908, vi, 251.

some extent at least by an internal genetic or biological constitution. Changes within the genetic constitution of living forms, if they are germ cells, are called mutations; if they are somatic cells (occurring especially in plant tissues) the changes are called somatic mutations. The conclusion is reached that somatic mutations may occur within the tumor cell, and that when these mutations do occur, they change the reaction potential and other physiological activities of the tumor cell. The data at hand are not sufficient to determine more in detail the nature of this mutational process. It may be either a change or shifting of a complete chromosome or chromosomes, or a change or changes within a chromosome or chromosomes (genic), or it may be even cytoplasmic in nature. By mutation I merely mean to use the term in its broadest sense; that is, a change or shift within the genetic or biological constitution that results in definitely clear-cut or discernible differences in behavior or structure that are perpetuated by the process of heredity (in this case cell division). It may be well to state however that the data at hand do not necessarily prove the presence of mutation. The conception of mutation does explain the phenomenon at hand—the other suggested explanations are certainly less probable—consequently it is justifiable to accept the conception of mutation in form of hypothesis.

We have, therefore, in the analysis of the data obtained in this experiment, an explanation of the phenomenon of the so called adaptiveness or virulence of the tumor cell. If the genetic change such as a somatic mutation is responsible for the change in the reaction potential of the transplantable tumor cell and other physiological activities, such as the proliferative vigor, then it is highly probable that the real cause of the phenomenon has been determined. A tumor increases its proliferative vigor or manifests an increased adaptiveness as a result of this mutational process. This somatic mutation may occur within a single cell in the neoplastic tissue. Then by the process of selective vitality or adaptiveness of the cells in the mass, the new type of behavior is obtained.

One other point I desire to mention. If one assumes that the spontaneous tumor possesses the same genetic constitution as the host tissue that gave rise to it (and we have every reason to suppose that it does have, since the tumor has arisen by some process or other from

cells derived from the body of the normal individual), then we must conclude, from our present data, that a tumor may deviate from the genetic constitution of the host tissue from which it arose, at least during the process of transplantation.

CONCLUSIONS.

1. Mutations or hereditary genetic changes may occur within the tumor cell at least during the process of transplantation.

2. These mutations may explain the phenomenon of the change in the reaction potential and deviations in the proliferative vigor of the tumor cell during the process of transplantation.

3. The nature of this mutational process is as yet undetermined.

4. The phenomenon of tissue specificity is determined to a large extent at least by the genetic constitution of the neoplastic tissue.

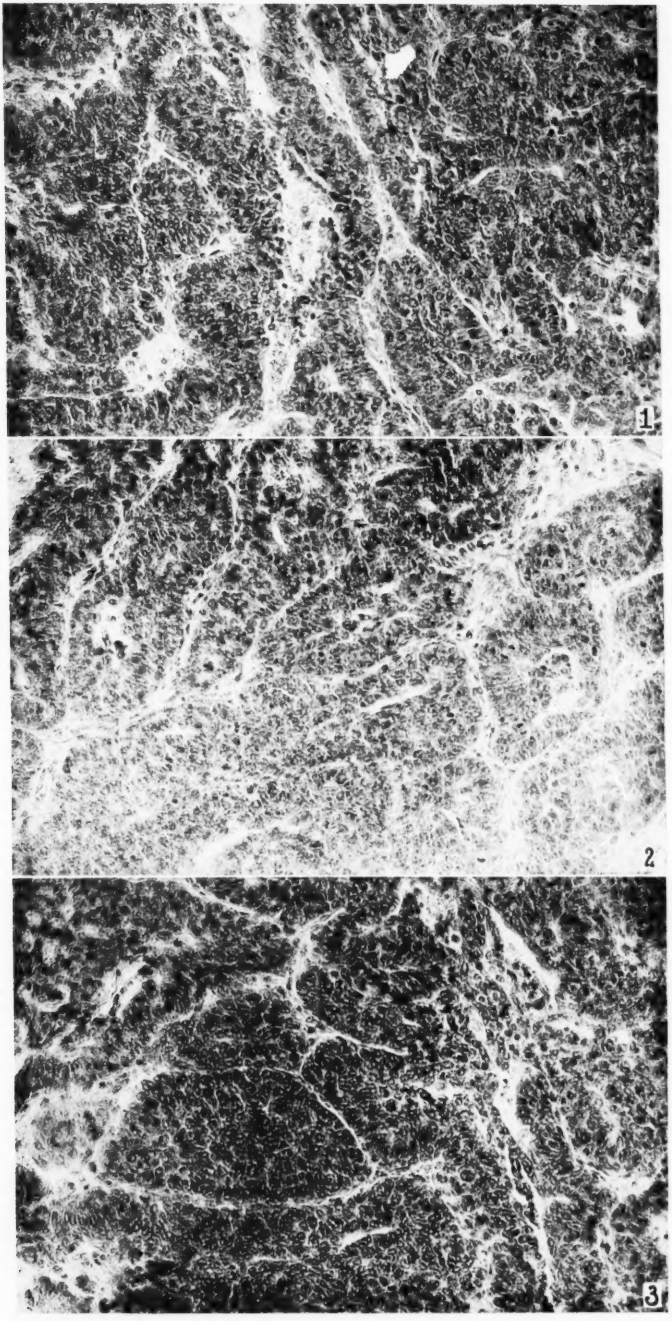
5. The tumor mass may deviate from the genetic constitution of the host tissue that gave rise to it, at least during the process of transplantation.

EXPLANATION OF PLATE 27.

FIG. 1. Photomicrograph of the original tumor, dBrD, used in Experiment 1. The tumor gave a 5.7 Mendelian factor ratio when inoculated into a series of F_2 individuals.

FIG. 2. A tumor transplant (dBrDm) derived from the original tumor, dBrD, given in Fig. 1. dBrDm gave a 2 factor ratio when inoculated into the same series of F_2 individuals used in Experiment 1 (Experiment 2).

FIG. 3. Another tumor transplant (dBrDBs) derived also from the original tumor, dBrD, during the process of transplantation. This tumor shows a 1 factor ratio when inoculated into a similar series of F_2 individuals (Experiment 4).



(Strong: Reaction potential of transplantable tumor.)

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